Anti-genotoxic effects of crude garlic extract on cisplatin induced toxicity on germ cells and morphology of sperms in *in vivo* mouse

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The present investigation was directed to study the possible anti-genotoxic activity of orally administered crude garlic extract (GE) against cisplatin-induced genotoxicity in mouse by using chromosomal aberrations (CA’s) in germ cells and sperm morphology essay (SMA). Three different doses of garlic extract (125, 250, 500 mg/kg) were tested for their modulatory capacity on the mutagenicity exerted by CP (10 mg/kg, i.p.). GE alone did not induce any significant variation in the incidence of CA’s and frequency of aberrant sperms. Pretreatment of mice with GE for 7 days and simultaneously with a single dose of Cisplatin significantly reduced the frequency of CA’s and frequency of aberrant sperms, suggesting that the garlic extract modulates the CP induced genotoxicity in a dose dependent manner.

**Key words:** Garlic, chromosomal aberrations, germ cells.

**INTRODUCTION**

cis-Dichlorodiamminoplatinum-II (CP) is an established anticancer drug used for the treatment of a variety of animal tumors and human cancers (Calabresi and Parks, 1985; Greene, 1992) such as ovarian, cervical, germ cell tumors and testicular teratoma (Muggia, 1984; Magdy, 2003; William and Christopher, 2009). The majority of antineoplastic drugs besides their generic growth property display genotoxic effects which in turn contribute to growth inhibition. These genotoxic effects may lead to initiation of unrelated tumours years after the cessation of chemotherapy (Beretta, 1991) by generating free radicals (Weijl et al., 1998; Wozniak et al., 2004) and also responsible for secondary malignancies observed in animals and some cured patients treated with CP (Kemph and Ivanovic, 1986; Greene, 1992; Pillaire et al., 1994). Much attention has been focused to reduce the mutagenic side effects of cisplatin by administration of modulating agents, usually free radical scavengers.

Garlic exhibits various beneficial effects like antitumor, antimutagenic, antioxidant and anticancer properties (Amagase, 2006; Banerjee et al., 2003; Kyo, 2001; Khanum et al., 2004; Lau, 2001; Sato and Miyata, 2000). One of the most important biological effects observed recently with garlic is tumor inhibitory properties against various types of cancers (Belman, 1983; Spannine et al., 1988; Fukushima et al., 1997). Hence, the present study is undertaken to evaluate the potential protective effects of orally administered garlic extract against Cisplatin-induced genotoxicity towards mouse chromosomal aberrations (CA) in germ cells and on morphology of sperms as an *in vivo* model.

**MATERIALS AND METHODS**

**Chemicals**

Trisodium citrate (Merck), NaCl (Loba Chemie), Methanol (s.d fine chemie), acetic acid (Qualingens), and 2% Giemsa stain solution in phosphate buffer (pH 6.8) were all purchased from E. Merck, India. Cisplatin, garlic extract, ethanol, mitomycin-C and eosin were purchased from Cipla. However, all other chemicals used in the experiments were of analytical grade.

**Preparation of garlic extract**

Fresh garlic bulbs (*Allium sativum* L.) purchased from the local market Dried and ground bulbs were submitted to extraction with 500 ml ethanol in a soxhlet apparatus for 72 h. After extraction, the solvent was filtered and made to evaporate by Rotavapor. The
obtained garlic alcoholic extract was stored at -20°C until use (Khalid, 2009).

Experimental animals

Eight weeks old randombred male Swiss albino mice (Mus musculus) average body weight of 25 ± 2 gms were purchased from National Institute of Nutrition, Hyderabad, were maintained in the departmental animal house under an absolute hygienic conditions as per the recommended procedures by fulfilling all the necessary ethical standards. They were housed in polypolyrene shoe box type cages dimensions were 13.5” L x 7.0” W x 6.5” to 8.5”H cages, bedded with rice husk (rice husk procured locally and autoclaved to free from micro organisms) and kept in air-conditioned room at the temperature 25°C (± 2°C) and RH 65 ± 5% and a photo-cycle of 12:12 h light and dark periods, were fed with pelleted diet (purchased from National Institute of Nutrition, Hyderabad, composed of 20.0% crude protein, 4.0% crude fiber, 1.0% calcium, 0.6% phosphorus, 8% fish meal, 20% ground nut cake and enriched with stabilized vitamins A, B, C, D, K, thiamine, riboflavin, pantothenic acid, niacin, folic acid, minerals and trace elements) and water ad libitum.

Treatment

Garlic (125, 250 and 500 mg/kg) extractions were given in orally for 7 consecutive days and 10mg/kg of Cisplatin was administrated on day 7 one hour after regular exposure to antimutagen as a single intraperitoneal dose. This is repeated for four weeks. Control (H2O) and positive control (0.1 ml of mitomycin-C) group of animals were also maintained simultaneously. 5 animals were used in each treatment and control group. Slides were screened with Leica CW 4000 Image analyzer.

Chromosome aberration analysis from germ cells

The mice were killed on 28th day, 24 h after administration of last dose of the drug. Seminiferous tubules from testis were collected in 5ml of isotonic 1.2% trisodium citrate solution and incubated at the temperature 37°C for 45 min. The cell suspension was centrifuged in 120x17 mm conical centrifuge tubes for 10 min at 1000 rpm. To the pellet 5 ml of freshly prepared pre-chilled fixative (3:1 methanol and acetic acid) added and centrifuged. This process repeated for 4 to 5 times. The Chromosomal preparations were made by the air drying technique (Evans et al., 1964) and stained with 2 ml of 2% Giemsa (2 ml of 2% Giemsa in 46 ml of double distilled water plus 2 ml of phosphate buffer pH 6.8) for 7-8 min. Approximately 500 meiotic metaphases screened for numerical (Autosomal Univalents, Sex- Autosomal Univalents, euploids and aneuploids) and structural (translocations) Aberrations.

Sperm morphology assay

All the control and treated animals were sacrificed on 35th day. This is because somatogenesis takes about 34.5 days to complete in mice. Sperms were sampled from the caudal epididymis after the animals had been sacrificed by cervical dislocation. Sperm suspension was prepared from the caudal of each testis by mincing the caudal in physiological saline. To the suspension 2-3 drops of 1% aqueous eosin was added and kept for about 20 min undisturbed. Smears were made on clean slides and allowed to dry in air. 1000 sperm cells/mouse were assessed for morphological abnormalities according to the criteria of Wyrobek and Bruce (1975).

Evaluation of sperm morphology essay (SMA)

The results obtained from the Sperm morphology essay are presented in Table 2. Treatment of mice with GE did not affect the parameter studied as compared to the control value. The percentage of sperm head abnormalities were increased after the administration of 10 mg/kg of Cisplatin. The increase was significant at the dose tested (P < 0.01). These findings agreed with those of Nereseyan et al. (2004) and Misra and Choudhury (2006). But a significant dose dependent reduction in the sperm head abnormalities was observed in GE primed animals.

Many fruits and vegetables are known to prevent chromosomal and DNA damage in animals (Ito et al., 1986; Miyata et al., 2004). It is due to many biologically active compounds which can trap the aggressive metabolites of mutagens. Several studies in the recent years have shown the antigenotoxic and antimutagenic effects of garlic for various drugs and chemicals (Shukla and Tanjea, 2002; Bhuvaneswari et al., 2004; Siddique and Afzal, 2005a; Belloir et al., 2006). Studies of the anticarcinogenic effects of garlic on several carcinogens were found to be effective in different ways such as antioxidant property (Khanum et al., 2004; Lampe, 2003).

Statistical analysis

The significance in the difference between control and treated groups was statistically analyzed by using χ² test. Data are expressed as mean ± SES in the Tables 1 and 2. Results were considered statistically significant at P < 0.05 (Bliss, 1967).

RESULTS AND DISCUSSION

Evaluation of chromosomal aberrations

The present study showed that the cisplatin significantly (P < 0.05) increased the chromosomal aberration frequencies from germ cells. These results are similar to the results observed by Adler and el-Tarras (1990), Choudhury et al. (2000) indicating the genotoxicity of this drug for germ cells. The results obtained in the present study showed that GE did not induce any increase in the incidence of CA’s as compared to the control value. These findings clearly indicates that GE did not have genotoxic effect at the doses tested (125, 250 as well as 500 mg/kg of GE for 7 consecutive days). These observations obtained are agreeable with that of al-Bekaii et al. (1990) who observed the increase in the weight of seminal vesicles and epididymides of male animals after the administration of Allium sativum in drinking water (100 mg/kg/day) for three months. Pretreatment of mice with GE for 7 consecutive days after cisplatin treatment significantly decreased the percentage of abnormal metaphases. These results are comparable with that of Kikelomo et al. (2008).

The results obtained from the Sperm morphology essay are presented in Table 2. Treatment of mice with GE did not affect the parameter studied as compared to the control value. The percentage of sperm head abnormalities were increased after the administration of 10 mg/kg of Cisplatin. The increase was significant at the dose tested (P < 0.01). These findings agreed with those of Nereseyan et al. (2004) and Misra and Choudhury (2006). But a significant dose dependent reduction in the sperm head abnormalities was observed in GE primed animals.

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Table 1. Effect of different concentrations of garlic extract on CA’s induced by CP in germ cells of mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal metaphases scored (%)</th>
<th>Abnormal metaphases scored (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>485 (97.00)</td>
<td>15 (3.00)</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>426 (85.20)</td>
<td>74 (14.80)</td>
</tr>
<tr>
<td>10 mg/kg Cisplatin</td>
<td>419 (83.80)</td>
<td>81* (16.20)</td>
</tr>
<tr>
<td>Garlic extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>125 mg/kg</td>
<td>482 (96.40)</td>
<td>18 (3.60)</td>
</tr>
<tr>
<td>250 mg/kg</td>
<td>479 (95.80)</td>
<td>21 (4.20)</td>
</tr>
<tr>
<td>500 mg/kg</td>
<td>476 (95.20)</td>
<td>24 (4.80)</td>
</tr>
<tr>
<td>Garlic + CP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/kg+125 mg/kg</td>
<td>443 (88.60)</td>
<td>57* (11.40)</td>
</tr>
<tr>
<td>10 mg/kg+250 mg/kg</td>
<td>464 (92.80)</td>
<td>36* (7.20)</td>
</tr>
<tr>
<td>10 mg/kg+500 mg/kg</td>
<td>483 (96.60)</td>
<td>17* (3.40)</td>
</tr>
</tbody>
</table>

*P<0.05

Table 2. Frequency of sperm head abnormalities recorded in cisplatin treated mice primed with garlic extraction.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal sperms scored (%)</th>
<th>Aberrant sperms scored (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4870±0.32 (97.40)</td>
<td>130±0.03 (2.60)</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>4685±0.03 (93.70)</td>
<td>315±0.09 (6.30)</td>
</tr>
<tr>
<td>10 mg/kg Cisplatin</td>
<td>4670±0.02 (93.40)</td>
<td>330*±0.1 (6.60)</td>
</tr>
<tr>
<td>Garlic extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>125 mg/kg</td>
<td>4860±0.03 (97.20)</td>
<td>140±0.1 (2.80)</td>
</tr>
<tr>
<td>250 mg/kg</td>
<td>4850±0.02 (97.00)</td>
<td>150±0.1 (3.00)</td>
</tr>
<tr>
<td>500 mg/kg</td>
<td>4840±0.02 (96.80)</td>
<td>160±0.08 (3.20)</td>
</tr>
<tr>
<td>Garlic + CP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg/kg+125 mg/kg</td>
<td>4760±0.03 (95.20)</td>
<td>240*±0.09 (4.80)</td>
</tr>
<tr>
<td>10 mg/kg+250 mg/kg</td>
<td>4790±0.03 (95.80)</td>
<td>210*±0.08 (4.20)</td>
</tr>
<tr>
<td>10 mg/kg+500 mg/kg</td>
<td>4810±0.02 (96.20)</td>
<td>190*±0.07 (3.80)</td>
</tr>
</tbody>
</table>

*P<0.05.

ability to scavenge free radicals, boosting the cellular antioxidants such as glutathione that prevent drug toxicity (Wei and Lau, 1998), increasing glutathione levels and blocking carcinogen binding to DNA (Amagase and Milner, 1993). Garlic is also a rich source of water- and lipid-soluble organosulfur compounds. Laboratory investigations have shown that both water- and lipid-soluble sulfur compounds from garlic provide its anticarcinogenic benefits.

In the present study, the frequency of CA’s in germ cells and sperm head abnormalities of mice treated with GE against Cisplatin showed the significant reduction. This reduction nearly reached the normal levels after treatment. The results obtained clearly indicate that the garlic extract modulates the CP induced genotoxicity in a dose dependent manner. Our results are in accordance with that of Assayed et al. (2008) in their study after administration of garlic extract for 5 consecutive days altered the abnormal spermatozoa parameters induced by cypermethrin in male white rats. Garlic also attenuated the adverse effects of testicular damage and spermiotoxicity induced by cadmium in rats (Kikelomo et al., 2008).

These findings proved that the consumption of antioxidants enriched diet can modulate the DNA damage caused by anti tumor agents which may be imparted to the ability of dietary antioxidants to trap free radicals generated by drugs. It is concluded that GE reduces the genotoxic action of CP in mice germ cells and sperm head abnormalities. Also in this study, all the selected doses of GE were found to be sufficient for the
modulatory effects. However, more detailed investigations in vivo are necessary before using garlic supplementation in chemotherapy.

REFERENCES


Crude Garlic Extract Modulates Cisplatin Induced Toxicity in In-Vitro Human Lymphocytes

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ABSTRACT:

Consumption of Garlic has been associated with reduced risk of many human cancers. Garlic (Allium sativum) has been used since ancient times, as a spice and also for its medicinal properties. The effect of crude ethanolic GE (GE) was studied experimentally on Cisplatin (CP) induced toxicity using 'chromosomal aberration assay and sister chromatid exchanges' in in vitro human lymphocytes. Three different doses of GE were tested for their modulatory capacity on the mutagenecity exerted by CP (0.12mg/ml). Cultures were harvested at 72hours. The results of the present investigations revealed that GE modulates the CP induced genotoxicity in a dose dependent manner.

Keywords: Cisplatin, Garlic, chromosomal aberrations, sister chromatid exchanges, human lymphocytes, in vitro.
1. INTRODUCTION

Cisplatin is one of the anti-cancer agents that falls into the DNA damaging agent class (Prasad and Giri, 1994). CP is generally considered to exerts its cytotoxic effect by binding its highly reactive hydrated platinum complex to DNA (Erickson et al., 1981; Roberts et al., 1988) resulting in mutation induction (Fichtinger-Scheppman et al., 1984). The compound is also highly toxic with a list of side effects including renal damage, severe nausea, vomiting, myelosuppression, ototoxicity and neurotoxicity (Lazar et al., 1978; Vermerken et al., 1982; Sorsa et al., 1985; Anthony et al., 1988; Fillastre, 1989; Abrams, 1990; Barbara et al., 1996; Nersesyan et al., 2003; Al-Etaby and Abou-Tarboush 2004; Thomas et al., 2004; Vijayalaxmi and Marie Prem D’Souza, 2004; Yingjun et al., 2008). Cisplatin posses another important side effect i.e reduction of antioxidant plasma levels and generation of free radicals in normal cells (Masuda et al., 1994; Baliga et al., 1998; Weijl, 1998; Wozniak et al., 2004) it is believed to be an important mechanism in the development of Cisplatin toxicity. It is also a potential human carcinogen it develops secondary malignancies in patients who has been treated with Cp (Greene, 1992).

Much effort has been put into reducing the mutagenic side effects of Cisplatin by administration of modulating agents, usually free radical scavenges. Free radical-mediated reactions are responsible for a wide range of chemotherapy-induced side effects, and antioxidants are able to protect non-malignant cells and organs against damage by cytostatic agents (Weijl et al., 1997). Recently a variety of compounds that possess antimutagenic properties has been detected in vegetables and spices, and evidence is accumulating that their dietary intake decreases the risk of cancer and other malignant diseases in human (Kada et al., 1986). The aim of the present study was to investigate the action of the antioxidant Garlic on chromosomal damage induced by Cisplatin, by determining their effect on the frequency of CP-induced chromosomal aberrations in In Vitro Human Lymphocytes.

2. MATERIAL AND METHODS

2.1 Chemicals:

RPMI 1640 medium, new born fetal calf serum and phytohaemagglutinin – M were purchased from Gibco. 5 – Bromo – 2 – deoxyuridine and Hoechst 33258 stain (40 Dg ml-1) from Sigma Aldrich, Colchicine from Loba-Chemie, 2% Giemsa stain solution in phosphate buffer (pH 6.8) from E. Merck, India, Cisplatin from Cipla India were obtained. All other chemicals used were of analytical grade.

2.2 Preparation of Garlic Extract:

Fresh Garlic bulbs (Allium sativum L.) were purchased from the local market and dried. The dried part of the bulb was made into coarse powder with mortar and pestle. The powder (about 250g) was soaked in 500ml of ethanol for 72 hours. The solvent was runned through rotavapour to separate solvent and concentrated through Soxhlet apparatus. The obtained garlic alcoholic extract was runned through rotavapour for further concentration. Final extract was lyophilized to powder form and stored at 4°C until use (Khalid and Al-Numair, 2009, Bozin et al., 2008).

2.3 Human lymphocyte culture:

The heparinized (100 units/ml) blood samples were obtained from healthy donors under aseptic conditions, with no recent history of exposure to mutagens. For each culture, the blood samples (0.5 ml) were placed in a sterile culture vial containing 5 ml of RPMI 1640 medium supplemented with 1 ml of fetal calf serum, antibiotics, 0.1 ml of phytohaemagglutinin and incubated at 37°C. Control cultures were also maintained simultaneously. At 70h after initiation, all treated and cultures were terminated by the addition of colchicines (0.05% to each vial) to arrest the cell
cycle at metaphase. After 2h of colchicines treatment the cultures were harvested and screened for various types of aberrations.

Various doses of GE 1.5, 3, 6 mg/ml and 0.12 mg/ml of Cisplatin were added separately to the culture vials. For modulation antimutagen was added at the initiation of the culture and Cisplatin was added on 2nd day. Cultures were harvested on 5th day, which corresponds to 72hours exposure to Cisplatin respectively. Each sample was maintained in quadrates.

2.4 Chromosomal aberrations analysis:

Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 5 ml of pre-warmed (37°C) 0.075 M KCl hypotonic solution was added to the cultures and incubated at 37°C for 20 min. After hypotonic treatment the cultures were centrifuged and the supernatant was removed, cells were fixed by adding chilled fixative (Methanol: Acetic acid; 3:1). The slides were prepared by air drying method method described by Moorhead et al., (1960) and stained with giemsa (2%) stain for 20 min. Later the slides were screened for chromosomal aberrations. For each concentration of treated and control groups 400 metaphases were scored.

2.5 Sister chromatid exchanges analysis:

For SCE analysis, 5- Bromo-2-deoxyuridine (3 µg ml-1) was added at the initiation of the culture. Mitotic arrest was done two hours prior to harvesting by adding 0.05% of colchicines. Hypotonic treatment and fixation were done in the same way described for CA. The slides were processed according to the method described earlier by Perry and Wolff (1974). For each concentration 30 metaphases were scored for SCE in control and treated groups.

2.6 Microscopic Examination:

Slides were prepared from all the cultures of different doses. The slides were stained with 2% giemsa (2ml of 2% giemsa and 2ml of phosphate buffer added to 46 ml 0f DDW). Air dried slides were coded and screened for the presence of various types of chromosomal aberrations and sister chromatid exchanges. 400 well spread; non overlapping metaphases were studied per concentration.

2.7 Statistical analysis:

The data on CA was analyzed statistically using 2x2 contingency Chi – Square test. Student’s t – test was used for calculating the statistical significance in SCE.

3. RESULTS AND DISCUSSION

The suitable method adopted for studying cytogenetic effects induced by a suspected agent in human beings is the micro culturing of human peripheral blood lymphocytes. It is an essentially important and sensitive indicator for both in vivo and in vitro induced structural and numerical aberrations. The results of the present study the incidence of chromosomal aberrations (CAs) were depicted in table-1 and SCEs were depicted in Table 2.

In the present work, cultures treated with CP (0.12 mg/ ml) had a higher frequency of CAs than the controls and dose dependent decrease was observed when treated with Garlic extract. Maximum decrease in CAs were found at the higher dose of Garlic extract rather than with the lower doses. The decrease in CAs were statistically significant (p<0.05) for 1.5, 3, 6 mg/ml of GE primed groups in comparison to CP alone treated lymphocytes. For the GE alone treated group, the percentage of aberrations did not show any significance level of changes. For the CP alone treatment, the percentage of aberrations scored 17.50 which decreased to 12.5, 8, 4.5 in Garlic primed cultures. This decrease in CAs was statistically significant. The CAs is one of the widely used parameters for testing the protective effects of natural compounds on the drug and chemical induced toxicity. The modulatory effect of natural compounds on the CAs...
induced by various kinds of chemicals and drugs is well established (Shukla and Taneja, 2002; Bhattacharya et al., 2004; Siddique and Afzal, 2005a; Dutta et al., 2007; Madhavi et al., 2008; Sowjanya et al., 2009).

Sister chromatid exchanges (SCEs) are symmetrical exchanges between newly replicated chromatids and their sisters. While homologous recombination may be one of the principal mechanisms responsible for SCEs. The SCEs are also used as one of the parameters for testing the protective effects of natural compounds on drug and chemical induced genotoxicity. In our experiments, the GE alone showed a dose dependent increase in the SCEs. There was increase in mean SCEs per cell when the concentration of GE increased from 1.5 to 6mg/ml. But such mean increase was not statistically significant. On the contrary, in the priming experiments, when the cells were treated with both CP and GE, there was a significant decrease in SCEs was observed. For the CP alone group, the mean SCE per cell was scored 7.43 which decreased to 2.50, 2.83 and 3.10 when primed with Garlic extract.

CP is a potential human carcinogen (Greene, 1992). The genotoxic action of the CP in human lymphocytes has already been reported by several authors like Turnbull et al.,(1979); Wiencke et al.,(1979); Morrison et al.,(1981); Pleskova et al.,(1984); Tandon and Sodhi (1985); Tofilon et al.,(1985); Blasko et al.,(1987); Kliesch and Adler (1987); Adler and El-Tarras (1989); Ohe et al.,(1990); Osanto et al.,(1991); Krishnaswamy and Dewey (1993); Choudhury et al.,(2000); Jin and Ikushima, (2004); Anuradha and Rudrama Devi, (2009) who observed a significant and dose-dependent effect for chromosome aberration formation and sister-chromatid exchanges in cultured mammalian cells, mouse bone marrow cells, and peripheral blood lymphocytes of patients.

Numerous scientific reports imply that vegetable intake may affect cancer incidence. Animal and in vitro studies provide evidence of the anticarcinogenic potential of several bioactive compounds in Allium vegetables (Wargovich et al., 1996). Potential anticarcinogens in Garlic have been identified and animal experiments suggest neoplastic inhibition from several Garlic-derived compounds (Bilyk and Sapers, 1985; Hughes and Lawson, 1991; Hertog et al., 1992; Dorant et al., 1993, Reddy et al., 1993, Ip et al., 1996; Sundaram and Milner, 1996; Wargovich et al., 1996; Schaffer et al., 1997; Sivam et al., 1997; Jonkers et al., 1999).

Present study clearly indicate that GEmodulates the CP induced genotoxicity in a dose dependent manner in human lymphocytes in vitro. Our results are comparable with the earlier published works where the GE has showed a significant decrease in the CA and SCE induced by various chemicals and drugs (Hageman et al., 1997; Bhattacharya et al., 2004; Siddique and Afzal, 2004, 2005a, 2005b; Belloir 2006; Yadav et al., 2006; Sowjanya et al., 2008; Sowjanya et al., 2009). Chemoprotective effects of GE against CP toxicity were reported earlier in animal models (Bianchini and Vainio, 2001; Shukla and Taneja, 2002; Premkumar et al., 2004). The protective and modulatory effects of the GEagainst the DNA damage, carcinogen bioactivation, and DNA adduct formation induced by the other compounds have been reported earlier on different cell lines (Hageman et al., 1997; Milner, 2001; Belloir et al., 2006). The modulatory response of Garlic may be attributed to the action of free radical scavenging, increasing the activity of antioxidant enzymes and inhibit the DNA adducts formation influencing the repair mechanism and modulating several metabolizing enzymes like cytochrome p450 and GST’s (Bianchini and Vainio, 2001; Khanum et al., 2004). Allicin, the main organic allyl sulfur compound in Garlic, exhibits strong antioxidant activity (Rabinkov et al., 1998). And also Garlic is a rich source of water- and lipid-soluble organosulfur compounds. Laboratory investigations have shown that both water- and lipid-soluble sulfur compounds from Garlic provide its anticarcinogenic benefits (Hussain et al., 1990; Perchellet et al., 1990; Rao et al., 1990; Sumiyoshi and Wargovich, 1990; Ip et al., 1992; Liu et al., 1992; Reddy et al., 1993; Schaffer et al., 1997).

In conclusion, the findings from the present investigation highlight the importance of commonly consumed dietary agents which are antigenotoxic. Additional studies are required before definitive conclusions can be drawn about the role of Garlic and Garlic supplements in cancer etiology.
Table 1: Effect of different concentrations of garlic extract on CA induced by CP in the cultured human lymphocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chromatid aberrations</th>
<th>Isochromatid aberrations</th>
<th>Number Polyploidy cells</th>
<th>Percentage of aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gaps</td>
<td>Breaks</td>
<td>Acentric fragments</td>
<td>Gaps</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>CP (0.12 mg/ml)</td>
<td>21</td>
<td>16</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Garlic Extract</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 mg/ml</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3 mg/ml</td>
<td>7</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>6 mg/ml</td>
<td>7</td>
<td>6</td>
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<td>2</td>
</tr>
<tr>
<td>Garlic + CP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 + CP</td>
<td>15</td>
<td>12</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>3+ CP</td>
<td>11</td>
<td>8</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>6+ CP</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

*Significant at P < 0.05 level    **P > 0.01

Table 2: Effect of Garlic extract on SCEs induced by CP in the cultured human lymphocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No of metaphases scored</th>
<th>Total No. of SCE’s scored</th>
<th>Mean SCE’s per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>15</td>
<td>0.50</td>
</tr>
<tr>
<td>CP (0.12 mg/ml)</td>
<td>30</td>
<td>223</td>
<td>7.43**</td>
</tr>
<tr>
<td>Garlic Extract</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 mg/ml</td>
<td>30</td>
<td>19</td>
<td>0.63</td>
</tr>
<tr>
<td>3 mg/ml</td>
<td>30</td>
<td>21</td>
<td>0.70</td>
</tr>
<tr>
<td>6 mg/ml</td>
<td>30</td>
<td>24</td>
<td>0.80</td>
</tr>
<tr>
<td>Garlic + CP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 + CP</td>
<td>30</td>
<td>73</td>
<td>2.50*</td>
</tr>
<tr>
<td>3+ CP</td>
<td>30</td>
<td>85</td>
<td>2.83*</td>
</tr>
<tr>
<td>6+ CP</td>
<td>30</td>
<td>93</td>
<td>3.10*</td>
</tr>
</tbody>
</table>

*P<0.05    **P>0.05

5. REFERENCES


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Modulatory Effects of *Murraya Koenigi* Leaf Extract on Cisplatin Induced Micronuclei in Bone Marrow Erythrocytes

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**ABSTRACT:**

The present study was undertaken to observe the chemoprotective effect of *Murraya Koenigi* leaves methanoloic extract against cisplatin induced cytogenetic damage in bone marrow erythrocytes of mice. Two experiments were conducted. In the first experiment animals were fed with 100,150,250mg/kg body weight of *Murraya Koenigi* extracts for 7 day orally. *Murraya Koenigi* Leaf extracts(MLE) treatment has not showed any mutagenicity indicates that the extracts are non mutagenic. In the second experiment, four groups of animals were maintained. When *Murraya Koenigi* leaf extract pretreated and concurrent administration of cisplatin 10mg/kg single dose intra peritonially reduced the frequency of micronuclei in polychromatic erythrocytes when compared with cisplatin induced significant incidence of micronuclei was observed. The P/N ratio was also reached to normalicy in pretreated MLE extract group of animals. Thus the results clearly indicate administration of MLE extract gave a significant protection in cisplatin induced genotoxicity and it is positive cytotoxic modulator chemotherapeutic strategy.

**Key Words:** Murraya Koenigi Leaf Extract, Cisplatin, Micronuclei, Protection.
1. INTRODUCTION

Cisplatin is used for the treatment of various cancers such as ovarian, head, neck, bladder, cervical and lung cancer\(^1\). The compound is toxic with side effects as renal damage, severe nausea, vomiting, myelosupression toxicity. Induction of chromosomal aberrations (CA) in \textit{invivo} somatic and germ cells, DNA adducts, micronuclei in human skin fibroblasts, CA’s in chinese hamster ovary cells, dalton’s lymphoma cells, sperm head abnormalities in mice\(^2\)\(^-\)\(^8\) hence it is necessary to protect the normal cells, a natural antioxidants and prone to prevent the toxicity induced by chemical genotoxins.

\textit{Murraya Koenigi} family – rutaceae (English; curry leaf tree, hindi: metha tree neem, Sanskrit: nahinib) has been used in Indian recipie preparation since several centuries . It possess antioxidant properties. Further this plant had shown to relieve the pain in kidney, diarrhea, anti-diabetic, antifungal and anti bacterial\(^9\)\(^-\)\(^10\). Animals administered with dimethyl hydrazine hydrochloride has been studied\(^11\). So far no studies are reported about its chemopreventive effects in animals. Hence in the present investigation a study was undertaken to study the chemoprotective activity against cisplatin induced micronuclei in bone marrow erythrocytes of mice.

2. MATERIALS AND METHODS

2.1 Chemicals:

Cisplatin kindly provided by Director, MNJ Institute of oncology and Mytomycin-C from Biochem Pharma Limited. The chemicals used in the study are of analytical Grade.

2.2 Animals:

Six to eight weeks old male mice (\textit{Mus Musculus}) of Swiss albino mice weighing about 25-27 gms procured from National Institute of Nutrition, Hyderabad, were used in this study. The mice were housed in poly propylene cages in a well ventilated room and were provided with standard pellet diet (M/S Lipton India limited) and water adlabitum.

2.3 Collection of Plant:

The fresh leaves of \textit{Murraya Koenigi} were collected from the local market and identified by Professor M. Pratiba Devi of Botany Department. Fresh leaves were washed under tap water and shade dried and powdered. 50\% of methanolic extract of the powder (500gms) was prepared with the help of cold maceration, at room temperature for about 20 hrs shaking frequently. The extracts were filtered and concentrated with vacuum rotovapour at 4\degree c. The value of extract obtained was 86.321\% w/w on dry basis.

2.4 Dosage Schedule:

Two experiments were conducted. In the first experiment four groups were maintained to study whether the plant extract is toxic or not in bone marrow cells. Hence the group I received control saline whereas group II, group III & group IV were orally administered with doses of 100mg /kg/bw, 150mg/kg and 250mg/kg/wt for seven days.

In the experimental groups the group I as only control vehicle group II animals treated with 250 mg/kg/BW methanolic extract of MKL for 7 days . group III is cisplatin 10mg/kg single dose intraperitonially. Group IV \textit{Murraya Koenigi} leaf treated (250mg/kg /bW) pretreated +cisplatin 10mg/kg intraperitonially one day prior to last treatment.

2.5 Analysis of Micronucleus Assay:

All the animals were sacrificed 24hrs after the last treatment and femur bones were dissected out from control and treated group of animals in 0.9\% physiological saline. Cleaned and muscle was removed with tissue paper. The bone marrow cells were flushed in 5 ml of fetal calf serum, centrifuged at 3000rpm for 10 min. to the pellet 5ml of 1:3 methanol and acetic acid. After 15minutes, the smears were prepared on clean slides air dried and stained with May and Grunewald stain according to the method of Schmid\(^12\). For each animal, minimum 2000 polychromatic erythrocytes were screened for the presences of micronuclei corresponding normo chromatic erythrocytes were scored.
The presence of micronuclei in polychromatic erythrocytes was used as an indicator of genetic damage. The scoring was done separately for each animal and it was observed that there was no significant difference between individual animals of same group. The ratio of polychromatic to normochromatic erythrocytes was used to estimate the effect on the proliferative activity of bone marrow cells. The data from studies were analysed using student T test.

3. RESULTS AND DISCUSSION

The animals were treated with methanol extract of *Murraya Koenigi* of three doses showed a increase at all dose levels in polychromatic erythrocytes of mice. However the differences in the frequency of micronuclei; between control and treated groups were insignificant (P>0.05) (table 1). The P/N ratio is not changed and the values were observed equal to the control values.

There was significant increase in the frequency of micronuclei from in control (0.18%) to cisplatin treated groups(1.32%). Whereas the pretreatment with the methanolic extract of *Murraya Koenigi* results showed a reduction in the induction of micronuclei when compared with cisplatin alone (table 2). The P/N ratio was decreased in cisplatin treated animals but concurrent administration of *Murraya Koenigi* leaf extract brings the values to lower range (0.62%). This indicates the chemoprotective nature of the *Murraya Koenigi* leaves. It is used as flavours to curry and sambar preparation especially by south Indias and it is available economically cheaper. The difference in the frequency of micronuclei between the group III & Group IV showed stastically significant (P<0.01). Thus the data indicate MLE supplementation reduced the cytotoxicity induced by cisplatin.

The *in vivo* micronucleus test is one of the best methods to screen the clastogenic effects of chemicals and drugs. Using this procedure the mutagenecity of various alkylating agents, pesticides and drugs in swiss albino male mice has been reported. The results of the present study clearly demonstrates as chemoprotective property of *Murraya Koenigi* leaf extract. The data clearly indicates the antigenotoxic property at all doses tested. Kleisch and Alder 1987 reported that MN in bone marrow cells of mice treated with cisplatin induction of sister chromatid exchanges and chromosomal damage *in vivo* and *in vitro* test system. Induction of MN and granular chromatin condensation in human skin fibroblasts *in vitro* by cisplatin has been reported.

**Figure 1:** The presence of micronucleus in adrimycin treated animals

![Figure 1](image1.png)

**Figure 2:** The absences of micronucleus in MLE treated animals

![Figure 2](image2.png)
Table 1: Frequency of micronuclei in bone marrow erythrocytes of mice treated with various doses of extract of MLE extract

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MN in polychromatic cells</th>
<th>MN in Normochromatic cells</th>
<th>Micronuclei in total cells</th>
<th>P/N Ration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23/12610 (0.18%)</td>
<td>13/13110 (0.10%)</td>
<td>36/25720 (0.23%)</td>
<td>0.96</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>25/12810 (0.21%)*</td>
<td>16/13160 (0.12%)</td>
<td>41/25970 (0.15%)</td>
<td>0.94</td>
</tr>
<tr>
<td>150 mg/kg</td>
<td>29/12124 (0.22%)*</td>
<td>17/13324 (0.13%)</td>
<td>46/25444 (0.18%)</td>
<td>0.90</td>
</tr>
<tr>
<td>250 mg/kg</td>
<td>27/12320 (0.24%)*</td>
<td>16/12800 (0.13%)</td>
<td>43/26220 (0.16%)</td>
<td>0.96</td>
</tr>
</tbody>
</table>

P>0.05

Table 2: Frequency of micronuclei in bone marrow erythrocytes of animals pretreated with MLE+cisplastin

<table>
<thead>
<tr>
<th>Group/Dose</th>
<th>P</th>
<th>N</th>
<th>MN in Total cells</th>
<th>P/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21/12120 (0.18%)</td>
<td>12/12140 (0.10%)</td>
<td>33/13352 (0.24%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Group II PFE 250 mg/kg bw</td>
<td>29/12160 (0.24%)*</td>
<td>29/12650 (0.23%)</td>
<td>58/24320 (0.23%)</td>
<td>0.96</td>
</tr>
<tr>
<td>Group III 5 mg/kg Cisplatin</td>
<td>161/12250 (1.32%)*</td>
<td>52/23820 (0.22%)</td>
<td>27/36070 (0.59%)</td>
<td>0.51</td>
</tr>
<tr>
<td>Group IV 250mg MLE+Cisplatin 5 mg/kg</td>
<td>78/12600 (0.62%)*</td>
<td>71/16200 (0.43%)</td>
<td>149/28800 (0.51%)</td>
<td>0.78</td>
</tr>
</tbody>
</table>

P<0.01, Significant of data calculated of G-IV with G – III only

The column chromatography led to the isolation of 3 compounds SU-I, II, III from petroleum ether and chloroform extract. Thus compounds were found to have anti-inflammatory property in rank order of (SU II 60% SU III 58.72% SU I 57.36%) 19. Further these leaf extract improved the learning of aged mice in hypoxic condition at 300kg/mg and 500mg/kg of extract when pretreated for 15 days 20. Further Murraya Koenigi stem bark extract in ether (SU-II) showed anti-cancer activity. Whereas dry plant powder showed a compound showed significant anti-diabetic activity in streptoztocin induced rats. The present results are comparable with earlier studies that Murraya Koenigi can significantly decrease the chromosomal damage caused by cyclophosphamide. The acetone extract of the bark of Murraya Koenigii exhibited significant reduction in the ccl4 induced hepatotoxicity as it reduced the SGOT, SGPT alkaline phosphates and total billirubin21-23.

It is well known that consumption of fruits and vegetables is associated and are known to prevent chromosomal and DNA damage in animals 24,25. Usually antimutagens acting in rodents are active in human too26. Our results have a practical decline of genotoxic effects of cisplatin in cancer patients some health care workers as nurse and pharmaceutical plant workers handle this drug which may alternate the higher risks for development of secondary malignancy and for abnormal reproductive outcomes due to its antioxidant activity of Murraya Koenige leaf extract.
4. REFERENCE


Introduction
Cisplatin is one of the most widely used chemotherapeutic drugs. The antitumour property of this drug was reported by Rosenberg and coworkers in 1969. It is effective against a wide variety of animal tumors and human cancers (Calabresi and Parks, 1985) like ovarian, head, neck, bladder, cervical, testicular teratoma, non- small-cell lung carcinoma and lung cancers, both as a single agent and in combination with other agents (Muggia, 1984). CP is generally considered to exert its cytotoxic effect by binding its highly reactive hydrated platinum complex to DNA (Erickson et al, 1981, Roberts et al 1988) resulting in mutation induction (Fichtinger-Scheiman et al, 1984). Along with its therapeutic activity this drug is known for its toxic nature in different test systems (Miyamoto et al, 2007; Hannan et al, 1988; James et al, 1998; Jean et al, 2003; Zoukova et al, 2007; Kuglik et al, 1990; Krishnaswamy G, Dewey WC, 1993; Amin et al, 2006, Somani et al, 2000). It is also a potential human carcinogen it develops secondary malignancies in patients who has been treated with cp.
The present experiments were designed to investigate the effects of cisplatin on germ cells of Swiss albino male mice.

MATERIALS AND METHODS

Eight-week old healthy laboratory bred Swiss albino mice (*Mus musculus*) weighing 25±3g was maintained under standard laboratory conditions at temp 22±20°C relative humidity 50±10% and a 12h photoperiod. Commercial pellet diet and deionised water were provided by labitum. In the present study the air drying technique of Evans et al (1964) was employed to study the effect of cisplatin on germ cells of mice. The animals were injected intraperitoneally with three different doses 2.5, 5, 10mg/kg body weight of CDDP on every 1st day of the month. This process was repeated at monthly intervals for two months. The air dried preparations were made on 7th day i.e on 60th day of the last treatment. Meiotic preparations were prepared according to standard cytogenetic method Evans et al (1964).

CHROMOSOME ABERRATION ANALYSIS FROM GERM CELLS

All the sets of treated and untreated groups were maintained for two months to allow the germ cells to complete two cell cycles. Control and positive control group of animals were also maintained simultaneously which received 0.1ml of distilled H₂O and 0.1ml of mytomycin-C. 4mg/kg body weight of colchicine was given 2hrs before scarifying the animals. All the animals were sacrificed by cervical dislocation on 60th day, 24hrs after administration of last dose of the drug. Animals were dissected out for testis and kept in physiological saline (0.9%NaCl).Tunica albugenia, the membrane covering the testes was removed carefully and the tubules were transferred into Petri dish containing the hypotonic solution of 1.2% trisodium citrate. The tubules of the testes were teased with the help of a bent forceps to release the cells into the hypotonic solution. The cell suspension was collected in clean centrifuge tubes and incubated at 37°C for 45minutes. After incubation the tubes were centrifuged for 10minutes. The supernatant was discarded and to the pellet 5ml of freshly prepared pre-chilled fixative (3:1 methanol and acetic acid) was added and allowed to stay at room temperature for 10minutes. This step was repeated 4 to 5 times. Finally the cells were fixed in fresh fixative. 3 to 4 drops of cell suspension were dropped on clean grease free, prechilled slides. The slides were stained with 2% giemsa and were screened for the presences of various types of chromosomal aberrations like structural and numerical aberrations in control and treated groups. A total of 100 well spread metaphases were observed per animal.

STATISTICAL ANALYSIS

The difference for the incidence of sperm abnormalities between the control and treated groups were subjected to statistical analysis using 2×2 contingency chisquare(χ²) test. chisquare(χ²) test is employed when the observation of two groups namely normal and abnormal are effected by two factors such as control and treatments. The 2×2 contingency of chisquare(χ²) test takes actual values into consideration.
RESULTS AND DISCUSSION

Cytogenetic methods for clastogenic activity of environmental pollutants are an essential part of routine testing programmes. The in vivo cytogenetic analysis of chromosomal aberrations is one of the best methods to evaluate the clastogenic activity of chemicals, drugs and environmental pollutants. Analysis of diakinesis of first metaphase stage of meiosis is a suitable stage for detecting the chromosomal aberrations induced in spermatogonia of treated animals.

Cisplatin is an important chemotherapeutic agent that is noted for its activity against testicular germ cell cancer. The data on the genotoxic effects of cisplatin evaluated from germ cells of mice after administration of the drug was furnished in tables 1 and 2. These include changes in different types of chromosomal aberrations.

In the present study, the frequencies of total chromosomal aberrations showed an increase over the control values. The percentage of chromosomal aberrations in control group of animals were 3.00 and it has been increased to 13.20, 15.00 and 16.00 in 2.5, 5, 10 mg/kg body weight of cisplatin treated animals respectively. The results clearly indicate that gradual increase in the values according to the dose.

Albanese (1987) used direct and indirect methods for the detection of chemically induced chromosome damage in male germ cells. The direct methods assess chromosome damage in the dosed animals, but analysis is restricted to the dividing spermatogonia and spermatocytes. Indirect methods, chromosome damage is assessed in the F1 progeny of the dosed male and analysis covers all germ cell stages. Both methods can provide evidence of germ cell exposure.

The present results are comparable with that of Sawhney et al (2005) who proved a dose-dependent reduction in testicular weight due to germ cell loss was observed when Normal adult C57/Bl/6J mice were exposed to cisplatin. Cisplatin is effective in killing spermatocytes, and spermatids (Marvin et al, 1982). In another study Vawda Al and Davies AG. (1986) proved the effects of cisplatin on the mouse testis by gradual increase in the reduction of the resting primary spermatocytes after treatment with cisplatin.

Cisplatin produces male reproductive toxicity by targeting multiple cell types in the testis (Leydig cells, Sertoli cells, and germ cells) Kim Boekelheide (2005) and by activating numerous molecular pathways involved in germ cell life-and-death decision making (Andrew et al, 2005). Sertoli cell (which play an essential role in spermatogenesis) toxicity was well studied by Monsees et al (2001), Meistrich ML(1984) with cisplatin. Early activation of oncogenes was observed in human head-neck tumors after treatment with Cisplatin (Németh et al, 2002).

Adler ID and el-Tarras A (1989) recorded an average of 1.4 aberrations per damaged cell in germ cells after employing cisplatin to (101/E1 X C3H/E1) F1 mice. In another study of Adler ID and el-Tarras A (1990) they observed that an increase in aberrant
cells during leptotene with preleptotene being the most sensitive stage. The dose-response relationship for aberrant cells was linear on day 13 after treatment. They concluded that, like mitomycin C (Adler, 1976), cisplatin primarily caused aberrations during the premeiotic phase of DNA synthesis.

The number of crossovers per meiosis is relatively constant. Recombinational interference ensures that each chromosome pair has at least one crossover. Otherwise, larger chromosomes would have multiple crossovers, and smaller ones would often have none (Sym, M. and Roeder, 1994; Kaback et al, 1992). The overrepresentation of single recombinants implies that interference (or another mechanism) operates to distribute crossovers evenly between chromosomes, avoiding nonrecombinants that would lead to possible aneuploidy. The germ-line recombination was observed by William et al (1997), Kartalou et al (2001) in cisplatin treated male mice at the early pachytene stage of meiosis I. They observed an increase on all three chromosomes examined and established a regimen that nearly doubled crossover frequency.

Cisplatin-induced apoptosis in germ cells of the mouse testes was observed by Zhang et al (2001) and it was rapid, reaching a peak within days of cisplatin exposure (Seaman et al, 2003). The transmission of the cytogenetic effects induced by cisplatin from spermatogonia to sperm was observed by Choudhury et al (2000). This phenomina proves the long term side effects of the drug (Petersen et al, 1994; Seaman et al, 2003).

In summary, the positive results obtained in the present study, warrants that the cytogenetic damage caused to germ cells may transmit to future generations, may produce deleterious effects, hence precautions are necessary when given chemotherapy treatment, as cisplatin is used extensively for a variety of cancers. Further investigations are needed to evaluate in vivo genetic damage of this anti neoplastic drug.

REFERENCES


Miyamoto CT, Sant’Anna JR, Franco CC, Castro-


Table 1: Frequency of chromosomal aberrations recorded in germ cells of mice after treatment with various doses of cisplatin

<table>
<thead>
<tr>
<th>Dose(mg/kg) and duration of treatment (hr)</th>
<th>Normal metaphases scored(%)</th>
<th>Abnormal metaphases scored(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>485</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>(97.00)</td>
<td>(3.00)</td>
</tr>
<tr>
<td>2.5 mg/kg</td>
<td>434</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>(86.80)</td>
<td>(13.20)*</td>
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<tr>
<td>5 mg/kg</td>
<td>425</td>
<td>75</td>
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<tr>
<td></td>
<td>(85.00)</td>
<td>(15.00)*</td>
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<tr>
<td>10 mg/kg</td>
<td>420</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>(84.00)</td>
<td>(16.00)*</td>
</tr>
</tbody>
</table>

The values are in parenthesis are percentages

*P<0.01

Table 2: Classification of various types of chromosomal aberrations recorded in germ cells of mice analyzed after treatment with various doses of Cisplatin

<table>
<thead>
<tr>
<th>Dose(mg/kg)</th>
<th>Numerical Aberrations</th>
<th>Structural Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Autosomal Univalents</td>
<td>Sex-Autosomal Univalents</td>
</tr>
<tr>
<td>Control</td>
<td>3(0.06)</td>
<td>8(16.00)</td>
</tr>
<tr>
<td>2.5mg/kg</td>
<td>13(26.00)</td>
<td>18(36.00)</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>15(3.00)</td>
<td>20(4.00)</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>17(34.00)</td>
<td>23(4.60)</td>
</tr>
</tbody>
</table>

The values are in parenthesis are percentages
EFFECT OF CISPLATIN ON MORPHOLOGY OF SPERMS: AN IN VIVO MICE MODEL

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SUMMARY

Genotoxicity of platinum antitumor agent cisplatin (CP, Cis-dichlorodiammine platinum II) was investigated by using mouse in vivo test system. This study reports the effects of CP on morphology of sperms in Swiss Albino male mice. Three different doses of CP viz., 2.5, 5, 10mg/kg body weight were used to evaluate the sperm head abnormality. The drug was administered intraperitoneally to experimental animals. Sperm preparations were made after 35 days. Cisplatin induced high frequency of sperm head abnormality at higher doses and the effect was significant at all the 3 doses tested. Animals and human studies have shown that sperm anomalies can be used as indicators and in certain cases, dosimeters of induced spermatogenic effects (Wyrobek 1982).

Key words: genotoxicity, cisplatin, mice, abnormal sperms, in vivo

INTRODUCTION

platinum-derived drugs are playing an increasing important role in the treatment of a variety of neoplasms (Olivi et al., 1993). Cisplatin Parent compound of this class, is effective against a wide variety of animal tumors and human cancers (Calabresi and Parks, 1985) like ovarian, head, neck, bladder, cervical, testicular teratoma, non-small-cell lung carcinoma and lung cancers, both as a single agent and in combination with other agents (Muggia, 1984). CP is generally considered to exert its cytotoxic effect by binding its highly reactive hydrated platinum complex to DNA (Erickson et al 1981, Roberts et al 1988) resulting in mutation induction (Fichtinger-Schepman et al, 1984). The compound is also highly toxic with a list of side effects including renal damage, severe nausea, vomiting, myelosuppression, ototoxicity and neurotoxicity (Abrams 1990; Anthony et al., 1988, Barbara et al., 1996, Fillastre, 1989; K.K. Vijayalaxmi and Marie Prem D'Souza, 2004, Lazar et al., 1978, M.K. Al-Etab and F.M. Abou-Tarboush 2004; Nersesyan et al 2003, Sorsa et al., 1985; Thomas et al, 2004; Von Hoff et al, 1979; Vermerken et al1982; Yingjun et al., 2008). Cisplatin posses another important side effect i.e reduction of antioxidant plasma levels and generation of free radicals in normal cells (Weijl, 1998; Wozniak, 2004). It is also a potential human carcinogen it develops secondary malignancies in patients who has been treated with cp (Greene M H. 1992).
In view of the above findings, in this study we assessed genotoxicity of cisplatin on morphology of sperms of mice using sperm morphology essay as a cytogenetic parameter.

**MATERIALS AND METHODS**

8-10 weeks old Swiss albino male mice with an average body weight of 22-24g were maintained under standard laboratory conditions at temp 22°C ± 2°C, relative humidity 50 ± 10% and 12h photoperiod. Commercial pellet diet and deionised water were provided by labium.

The test compound cisplatin manufactured by cipla pvt ltd purchased form local pharmacy shops. NaCl from S.D fine chemie, Giemsa, Colchicine from Himedia. All other chemicals used in the present experiments were of analytical grade.

**SPERM MORPHOLOGY ASSAY**

In the present study on dose effect relationship the animals were injected intraperitonially with various doses of cp 2.5, 5, 10mg/kg body weight. Control group of animals were also maintained simultaneously. All the control and treated animals were sacrificed on 35th day by cervical dislocation after the administration of the test compound. Sampling time i.e., 35 days was used for this study. This is because the germ cells which are exposed at late spermatogonial stage to the chemical, would reach the cauda epididymis after undergoing a series of changes during the course of development to give rise to sperms. 5 animals were used in each treatment and control group. 0.2ml of colchicine (5mg/kg body weight) was given 2 hrs before scarifying the animals. Animals were dissected out for testes and both the cauda epididymis were removed and placed in a Petridish contaiing 0.9% NaCl (hypotonic) solution. The cauda epididymides were teased thoroughly to release the Sperms and stained with 1% aqueous eosin for about 20 minutes. A drop of the sperm suspension was smeared on a clean slide. One thousand sperms per animal were scored from each group for the presence of sperm shape abnormalities.

**STATISTICAL ANALYSIS**

The difference for the incidence of sperm abnormalities between the control and treated groups were subjected to statistical analysis using 2×2 contingency chisquare (χ²) test. chisquare (χ²) test is employed when the observation of two groups namely normal and abnormal are effected by two factors such as control and treatments. The 2×2 contingency of chisquare (χ²) test takes actual values into consideration.

**RESULTS AND DISCUSSION**

Cisplatin is an important chemotherapeutic agent that is noted for its activity against testicular germ cell cancer. As a heavy metal coordination compound, cisplatin produces DNA cross-links that are presumably responsible for its antineoplastic effect (Kartalou et al. 2009).
Chemotherapy with cisplatin can have profound and long-lasting effects on spermatogenesis (Petersen et al 1994).

The morphology of sperms serves as an important and sensitive indicator in assessing reproductive toxicology. They can be used to evaluate the spermatogenic damage, fertility and heritable genetic changes. In the present study sperms were analysed according to the criteria of Wyrobek and Bruce (1978). Sperm heads with out tails or heads, which are in contact with each other being overlaid with other sperms were excluded in the scorings. Different Types of abnormal sperms observed were amorphous, banana, hookless, folded, double headed and double tailed. Since the present investigation, among the above categories amorphous and hookless are mostly observed when compared to normal sperms.

In the present study, various doses 2.5, 5, 10mg/kg of the drug were used for the experiment. The frequencies of the abnormal sperms in controls were 2.80% when compared to that of treated groups were 10.00, 12.40 and 14.80%. The results clearly indicate that the highest dose of the drug has resulted in the increase of abnormal sperms and the effect was significant at all the three doses tested in the present investigation.

Cisplatin, a cancer chemotherapeutic agent that produces male reproductive toxicity by targeting multiple cell types in the testis. (Wyrobek et al, 2005) Meistrich et al (1982) demonstrated acute damage to spermatogenesis following intraperitoneal or intravenous injection of cisplatin at low doses and cisplatin was selectively toxic for spermatogonia, with an LD50 of 1.1 mg/kg. At higher doses, cisplatin had broad activity, killing some cells in all stages, including spermatocytes and spermatids in the adlumenal compartment. In another study Meistrich ML (1984) proved that activity against cells in the adlumenal compartment, which is normally protected by the blood-testis barrier, indicated that cisplatin might induce Sertoli cell toxicity.

Table 1: Frequency of sperm head abnormalities recorded in mice after administration with different cisplatin
dose.

<table>
<thead>
<tr>
<th>Dose(mg/kg)</th>
<th>Normal Metaphases scored(%)</th>
<th>Abnormal Metaphases scored(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4860</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>(97.20)</td>
<td>(2.80)</td>
</tr>
<tr>
<td>2.5 mg/kg</td>
<td>4500</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>(90.00)</td>
<td>(10.00)*</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>4380</td>
<td>620</td>
</tr>
<tr>
<td></td>
<td>(87.60)</td>
<td>(12.40)*</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>4260</td>
<td>740</td>
</tr>
<tr>
<td></td>
<td>(85.20)</td>
<td>(14.80)*</td>
</tr>
</tbody>
</table>

The values are in parenthesis are percentages *P<0.01

Cisplatin not only exhibit multiple cell toxicity, it also causes clastogenic effects in the differentiating spermatogonia in mice (Adler and el-Tarras 1990). According to their report spermatogonial cells are less sensitive than the bone marrow cells. However, in other studies cisplatin induced significant clastogenic effects in spermatogonia also. Induction of sex-linked recessive lethal mutation in male germ cells (Woodruf et al. 1980) and clastogenicity in spermatocytes and spermatogonia (Brodberg et al. 1983) in Drosophila melanogaster by cisplatin have been reported. Cisplatin induced significant aberrant spermatocytes, autosomal and sex chromosomal univalents, tetravalents etc., in male mice (Adler and el-Tarras 1990; Choudhury et al. 2000). Oshio et al 1990 studied the quality of epididymal sperm was evaluated by sperm count, motility, morphology of sperm, and the apparent density of sperm. At 10 mg/kg dose, about 80% mortality occurred during the administration period and also CDDP causes a reduction in sperm apparent density and impairs semen quality in mice. Ahmet Atessahin et al 2006 observed that decreased sperm concentration (p < 0.05) and sperm motility (p < 0.001), increased total abnormal sperm rates (p < 0.05) as compared with the control group rats Administration of cisplatin. Monsees et al 2001 studied the effect of various environmental pollutants, including pesticides, oestrogenic compounds and heavy metals on Sertoli cells, which play an essential role in spermatogenesis. They observed the significant decrease in Sertoli cell viability. Platinum compounds also reduced the sperm motility in humans (Kesseru and Leon 1974). Bruce et al. 1974 observed the significant reduction in the sperm count, indicating the toxic effects of cisplatin on spermatogenesis.

Various reasons are given for the induction of abnormal sperms in mice. Perhaps they are the result of naturally occurring errors in the differentiation process or the consequence of abnormal chromosomal compliment (Bruce et al. 1974). According to Topham (1980a and b), the characteristics controlling the sperm head shape are carried on autosomes and sperm abnormality test identifies those agents which cause small alterations to the testicular DNA. A more comprehensive study is required to understand the mechanism of the action of the drug.

REFERENCES


