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Studies on the Genotoxic Effects of Anticancer Drug Carboplatin in *in vivo* Mouse

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ABSTRACT Genotoxicity of platinum antitumor agent carboplatin (Cbp) was investigated by using mouse *in vivo* test system. Three different doses of carboplatin viz., 30, 60 and 90 mg/kg b.w were used for the experiment. Fetal liver micronucleus (MN) test and sperm abnormality assay were used as the test parameters. Cyclophosphamide (50 and 100mg/kg b.w) was used as positive control and distilled water formed the negative control. Both the chemicals were administered intraperitoneally to experimental animals. MN and sperm preparations were made at 24 hr. and 35 days respectively. Carboplatin induced significant MN both in the fetal liver and the maternal bone marrow at different doses. It also induced high frequency of abnormal sperms at higher doses and the effect was significant at all the 3 doses tested.

INTRODUCTION

The platinum antitumor agents are unique, coordination complexes and the parent compound of this class cis-diammine-dichloro-platinum (II) (cisplatin), contributes to the curative treatment of various cancers such as, testicular teratoma, ovarian, head, neck, bladder, cervical and lung cancers. The compound is also highly toxic with a list of side effects including renal damage, severe nausea, vomiting, myelo-suppression, ototoxicity and neurotoxicity (Abrams 1990). Since the antitumor properties of cisplatin were reported by Rosenberg and Coworkers in 1969, well over a thousand platinum analogues have been tested for varieties of antitumor activity. The aim of this type of work has been to find analogues with reduced toxicity and /or wider spectrum of activity. One such analogous compound is carboplatin. Carboplatin entered clinical trials in 1981 and showed a very similar activity profile to that of cisplatin, with good response in ovarian, small cell lung, head and neck and testicular cancers. It is currently the second most widely used platinum anticancer drug in the world (Sai Man Liu 1998; Martindale 2002). In the present study an attempt has been made to study the genotoxicity of this widely used antitumor agent. There are no reports on the *in vivo* genotoxicity of this compound which is an analogue of cisplatin but, there are few reports on the *in vivo* and *in vitro* genotoxicity of cisplatin.

In comparative studies cisplatin was reported to be several times more mutagenic than other tested platinum salts. Adler and el-Tarras (1989) reported the induction of chromosomal aberrations(CA) *in vivo* both in somatic and germinal cells treated with cisplatin. Platinum-DNA adducts were formed during post treatment incubation of *Escherichia coli* with cisplatin (Razaka et al. 1988). Induction of MN and granular chromatin condensation in human skin fibroblasts has been observed after cisplatin treatment (Jirsova and Mandys 1994). Cisplatin induced cell killing and CA have been reported in Chinese hamster ovary cells (Krishnaswamy and Dewey 1993). Sorsa et al. (1985) reviewed the potential and real hazards due to occupational exposure to anticancer drugs, including cisplatin and reported it as a positive genotoxic agent in prokaryotes as well as in eukaryotes. Prasad et al. (1998) studied the effect of cisplatin on the chromosomes of murine Dalton's lymphoma cells in *in vivo* bone marrow cells. Hannan (1988) made a comparative study of the cytotoxic and genotoxic effects of cisplatin and its analogue, TNO-6 in yeast.

Since there are very few reports on the *in vivo* genotoxicity of parent compound cisplatin and no reports on such effects of carboplatin present study was undertaken using mouse *in vivo* transplacental MN test and sperm abnormality assay. As an alternative to the classical method of chromosome aberration assay, analysis of the frequency of occurrence of micronuclei in treated

cells provides a comparatively rapid and sensitive indication of both chromosomal aberrations and chromosome loss that lead to numerical chromosomal anomalies. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome lagging at anaphase or from acentric chromosomal fragments (Schmid 1973). 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).

MATERIALS AND METHODS

Chemicals

The chemical is cis-diammine (cyclobutane-1,1,dicarboxylato) platinum, with chemical formula - $C_6H_{12}N_2O_4Pt$. and Molecular weight, 371.2 (Martindale 2002). Like in cisplatin in carboplatin also the platinum moiety that is, diammine-platinum binds to DNA, but carboplatin forms reactive aquated platinum species much more slowly than cisplatin. The half-life of carboplatin in chloride free phosphate buffer is 268 hours as opposed to 24 hours for cisplatin under the same conditions. This lower reactivity is manifested in lower potency and toxicity of carboplatin than cisplatin (Abrams 1990**).**

Carboplatin (Carbotinol ,CAS No.41575 – 94 – 4;) manufactured by Korea United Pharm. Inc., South Korea, purchased from VHB Pharmaceuticals Pvt. Ltd., Mumbai, was used for the experiments. Three doses of the drug, i.e., 30, 60 and 90 mg/kg . were selected on body weight basis. The highest dose was equivalent to therapeutic dose of 400 mg/m^2 in humans and this was converted into body weight dose using the method of Freireich et al. (1966). Different concentrations of Cbp were prepared by dissolving the drug in double distilled water and administered intraperitoneally to experimental animals as a single dose in 0.1 ml quantity.

In this experiment distilled water formed the negative control and cyclophosphamide (CP; CAS no. 6055-19-2, Endoxan- Asta, German Remedies Ltd., Goa, Batch no.G161) formed the positive control.

Experimental Animals

Swiss albino mice (*Mus musculus*) were used for the experiment. The animals (original stock was procured from Indian Institute of Sciences, Bangalore) were bred and maintained in the departmental animal house under an absolute hygienic condition as per the recommended procedures by fulfilling all the necessary ethical standards. They were housed in polypropylene shoe box type cages, bedded with rice husk and kept in air-conditioned room, at 25° C (± 2° C) and RH 65 ± 5 %, were fed with pelleted diet (Amruth Feeds, India) and water *ad libitum*. Animals with average body weight of 25±2 gms. were used for the experiment.

Transplacental MN Test

Fetal liver MN assay was done by using the method of Cole et al. (1979). Eight weeks old virgin male and female mice were used for the experiment. Each virgin female mouse was randomly mated with a male overnight and next morning after the mating was designated as gestation day 0. The pregnant females were treated with the chemicals on the day 14 of gestation. Three pregnant females were taken in each treated and control group. In this experiment positive control CP was used at a dose of 100 mg/kg b.w

On day 15 of gestation, i.e., 24 hours after the treatment with the test chemicals, the pregnant females were sacrificed by cervical dislocation. The abdominal region was exposed and four fetuses were removed from each animal (two from each uterine horn) and washed with physiological saline to remove the maternal and fetal blood. Then the fetuses were transferred to fresh saline and cut open to remove the fetal liver. Fetal liver was transferred to HBSS solution and thoroughly washed and was minced separately in 2 ml of 5% fetal calf serum (FCS) in HBSS (pH = 7.2) and were transferred to test tubes. The content of each tube was dispersed into a fine cell suspension by aspirating several times with a Pasteur pipette. The tube was left undisturbed for 5 minutes and then the upper portion of the cell suspension, free of tissue fragments was transferred to a centrifuge tube and centrifuged at 1000 rpm for 8 minutes. The supernatant was discarded and the pellet was suspended in a small amount of HBSS - FCS mixture. A drop of suspen-sion from each tube was smeared on clean glass slides and air dried. All slides were then fixed in absolute methanol for 5 minutes and dried.

The maternal bone marrow MN slides were also prepared simultaneously from the same animals using the modified method of Schmid

(1973) where 5% bovine serum albumin (BSA) was used as suspending medium for marrow cells (Seetharam et al. 1983) instead of fetal calf serum. After removing the fetuses, from the same animal femur bones were removed and marrow cells were flushed with BSA, centrifuged and marrow smears were made on clean slides. The slides were then fixed in absolute methanol and air dried. In both the cases, slides were stained with May Grunwald-Giemsa and scored for the presence of MN.

In transplacental MN test about 2000 PCE's and corresponding NCE's were scanned from each animal (500 PCE/fetus) for the presence of MN. Maternal bone marrow slides were also scanned for micronuclei in PCE as well as in NCE. P/N ratio was also determined in both the cases.

Sperm Abnormality Assay

Sperm shape abnormality assay was done following the method of Wyrobek and Bruce (1975). Same doses of carboplatin were used for sperm abnormality assay also. 8 weeks old male Swiss albino mice were used for the experiments. One post treatment sampling time i.e., 35 days was used for the 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. Initial body weight of the animals was taken before the start of the experiments and sacrifice body weight was taken on 35th day just before killing the animals. Animals were sacrificed by cervical dislocation and the testes were dissected out and weighed. Both the cauda epididymis were removed and placed in a watch glass containing 1 ml phosphate buffered saline (pH = 7.2). The cauda epididymides were minced thoroughly and the suspension obtained was filtered through a fine mesh cloth to remove tissue debris and stained with 1% aqueous eosin for about 20 minutes. A drop of the sperm suspension was smeared on a clean slide.

Two thousand sperms per animal were scored from each group for the presence of sperm shape abnormalities following the criteria of Wyrobek and Bruce (1975). Sperm count was done with the help of the haemocytometer. An aliquot (0.05ml) from the sperm suspension (1ml) was diluted 40 times (1:40) with PBS and mixed thoroughly. Diluted sperm suspension was introduced into the Neubaur counting chamber and the total sperm count in 8 squares of 1 mm^2 was determined and multiplied by 5 X 10^4 to calculate the number of sperms per epididymis (Vega et al. 1988)

Statistical analysis of the results obtained was done by using paired t –test (Hassard 1991) and Mann-Whitney – U test (Siegel 1956)

RESULTS

The results obtained for transplacental micronucleus test are presented in table1. Carboplatin at different doses induced significant MN in the fetal liver, both in PCE and NCE. There was a significant reduction in the P/N ratio in the Cbp treated groups compared to the negative control. In maternal bone marrow also significant frequency of MN were observed (Table 2).

Carboplatin induced high frequency of abnormal sperms at higher doses and the effect was significant at all the 3 doses tested. Types of abnormal sperms observed were amorphous, banana, hookless, folded, double headed and double tailed (Table 3). A significant dose dependent reduction in the sperm count was also observed in the Cbp treated animals which was again dose dependent (Table 4).

Table 1: Frequency of MN induced by carboplatin and controls in fetal liver at 24h.-Transplacental MN test.

Treatment	*Dose in mg/kg*	*% PCE*	*% NCE*	*%MN PCE^A±SEM*	*% MN NCE± SEM*	*P/N Ratio ± SEM*
D.W.	0	93.18	6.82	0.12±0.02	0.08±0.10	13.66±0.46
CP	100	84.22	15.78	3.65±0.04^c	8.17±0.45^c	5.34±0.13^c
Cbp	30	89.89	10.11	2.07±0.02^c	2.56±0.07^c	8.93±0.02^c
Cbp	60	89.67	10.33	2.14±0.05^c	6.07±0.31^c	8.72±0.03^c
Cbp	90	90.01	9.9	2.66±0.03^c	6.52±0.05^c	9.10±0.24^c

Paired t-test : ^{c} p > 0.001

^{A} – Three pregnant females /group, 500 PCE / fetus and 4 fetuses /animal.

Table 2: Frequency of MN induced by carboplatin and controls in maternal bone marrow at 24h. – Transplacental MN test.

Treatment	*Dose in mg/kg*	*% PCE*	*% NCE*	*% MN PCE[A] ± SEM*	*% MN NCE ± SEM*	*P/N ratio ± SEM*
DW	0	58.27	41.73	0.19±0.00	0.09±0.00	1.01±0.00
CP	100	29.6	70.4	2.83±0.04[c]	1.04±0.05[c]	0.42±0.01[a]
Cbp	30	47.5	52.52	1.12±0.04[b]	0.38±0.02[b]	0.84±0.01[b]
Cbp	60	45.64	52.46	1.65±0.02[c]	0.67±0.05[c]	0.76±0.01[b]
Cbp	90	43.2	56.8	2.88±0.04[c]	0.85±0.03[c]	0.42±0.06[c]

Paired t-test : [a] $p > 0.05$; [b] $p > 0.01$; [c] $p > 0.001$
[A] -Three pregnant females /group, 2000 PCE /animal

Table 3: Results of sperm shape abnormality assay with carboplatin and controls.

Treatment	*Dose mg/kg*	*Amorphous*	*Banana*	*Hookless*	*Folded*	*Double head*	*Double tail*	*Total*	*%Abnormal Sperms [A] ± SEM*
DW	0	33	8	31	26	2	7	107	1.07±0.26
CP	50	182	43	149	100	12	25	511	5.11±0.50[b]
Cbp	30	130	54	101	55	5	5	350	3.50±0.24[a]
Cbp	60	200	48	181	50	4	16	499	4.99±0.20[b]
Cbp	90	230	89	208	51	6	17	601	6.01±0.44[b]

Mann-Whitney U-test: [a] $p = 0.004$, [b] $p = 0.008$, [A] = 5 animals/group and 2000 sperms/animal

Table 4: Effect of carboplatin and controls on body weight, testes weight and sperm count in mice.

Treatment[A]	*Dose mg/kg*	*Initial b.w. (gms)*	*Sacrifice b.w. (gms)*	*Testes wt.(gms) ± SEM*	*Sperm count /epididymis ($x\ 10^6$) ± SEM*
DW	0	27	29.5	0.21±0.02	8.70±0.52
CP	50	24	27.2	0.19±0.03[a]	5.52±0.24[b]
Cbp	30	28.24	30.34	0.26±0.05	5.69±0.94[b]
Cbp	60	23.96	27.12	0.22±0.04	4.57±0.66[c]
Cbp	90	24.12	27.98	0.25±0.03	3.46±0.16[c]

Paired t- Test – [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$, [A] = 5 animals/group

DISCUSSION

In the present investigation we observed significant MN in fetal liver cells in carboplatin treated groups. This clearly showed the transplacental genotoxic effects of this drug. Although there are no reports on the genotoxicity of carboplatin, many reports are available on such effects of cisplatin.

Cisplatin is found to be a specific inducer of dominant lethal mutations in female mice and it causes different types of chromosomal damage and DNA adducts (Bloomaert et al. 1995). It produced genetic effects in rodent oocytes resulting in early embryonic mortality (Hooser et al. 2000; Katoh et al.1990). Cisplatin easily crosses the placental barrier and affects the fetus (Koc et al. 1994). Embryotoxicity and teratogenecity of cisplatin has been reported in mouse (Lazar et al. 1978; Sorsa 1985). Lazar et al. (1978) observed transplacental effects of cisplatin in Swiss Webster mice and various types of abnormalities were reported in fetuses delivered from dams treated with cisplatin. Cisplatin was also found to be embryotoxic. Even at a dose of 3 mg/kg it killed 50% of the exposed fetuses. The surviving fetuses showed weight reduction and skeletal malformations. Administration of these drugs during pregnancy exposes the fetus to platinum, leading to DNA adduct formation in utero (Henderson et al. 1993; Koc et al. 1994). Arnon et al. (2001) reviewed the genetic and teratogenic effects of cancer treatments on gametes and embryos. Cisplatin and analogues were also included in this review. Cole et al. (1979) have given a detailed description of cell kinetics and its role in the induction and incidence of transplacental micronuclei in mouse. The transplacental test has a particular advantage to elucidate the genotoxic effects of chemicals which arise from maternal or fetal metabolism

(Cole et al. 1979; Galloway et al. 1980; Nakamura et al. 1993).

There was significant induction of sperm abnormalities in mouse treated with carboplatin indicating germinal effects of carboplatin. Reports are available on the cisplatin induced germ cell effects. Cisplatin showed 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). Platinum compounds also reduced the sperm motility in humans (Kesseru and Leon 1974). In the present study significant reduction in the sperm count was noted, indicating the toxic effects of carboplatin 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.

Carboplatin induced significant MN in maternal bone marrow also. Many reports have shown the clastogenic effects of cisplatin. Kliesch and Adler (1987) reported the MN in bone marrow of mice treated with cisplatin. In this study cisplatin induced high frequency of MN even at very low doses (1-5 mg/kg). Induction of sister chromatid exchanges and chromosomal aberrations, *in vivo* and *in vitro* by cisplatin have been reported. Jirsova and Mandys (1994) studied the induction of MN and granular chromatin condensation in human skin fibroblasts in *in vitro* by cisplatin.

Carboplatin reduced the P/N ratio both in fetal liver and maternal bone marrow indicating the cytotoxic effects of this drug. Krishnaswamy and Dewey (1993) reported the cisplatin induced cell killing and chromosomal aberrations in CHO cells treated during G1 and S phase. Many of the anticancer drugs act as mitotic inhibitors by directly acting on the microtubules. Only the difference observed with different agents is the variation in their cytotoxicity.

Carboplatin and cisplatin are considered as miscellaneous alkylator like agents. The important strategy for cancer treatment is to use anticancer drugs with alkylating properties. The reaction of DNA with alkylating agents results in a covalently modified bases. The alkylating agents are classified as monofunctional, bifunctional alkylating agents and some others are topoisomerase inhibitors and some function as free radical generating agents. In all these cases, they ultimately attack the DNA molecules and bring about chromosomal changes. By using ^{14}C labeled ligand, carboplatin was shown to bind monofunctionally to DNA after which there was a time dependent formation of difunctional inter-strand cross links formed from some of these initially monofunctional adducts. The effects of Cbp and cisplatin on the DNA are the same once bound to the same extent. Equal binding of the two drugs to DNA in various cell systems resulted in equal cytotoxicity in *in vivo* (Knox et al. 1986). Tilby et al. (1991) observed DNA adducts induced by carboplatin and they recognized the similarity of adducts formed by cisplatin and carboplatin. DNA strand breaks and DNA cross links have been reported in peripheral mononuclear blood cells of cancer patients during chemotherapy with cyclophosphamide and carboplatin (Hengstler et al. 1992).

Alkylation of DNA has become a well recognized requirement for the mode of action of cytotoxic drugs. Therefore, the more recently introduced cancer chemotherapy drugs that is platinum derivatives became regarded as alkylating agents although chemically they are not. The platinum drugs showed a close analogy in the mode of action with mustard compounds, which were first introduced as anticancer drugs (Lawley and Philips 1996).

Present study proved the genotoxicity of carboplatin but the doses needed for such effects is higher than the cisplatin. Therefore, it could be concluded that this drug is less toxic than cisplatin as far as genotoxicity is concerned.

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