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The Effects of Cisplatin on Murine Metaphase II Oocytes

RUTH E. HIGDON, M.D.,[1] FRANCESCO MARCHETTI, D.SC., JOHN B. MAILHES, PH.D., AND GEORGE L. PHILLIPS, M.D.

Louisiana State University Medical Center, Division of Gynecologic Oncology, Shreveport, Louisiana 71130-3932

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This study was undertaken to determine if cisplatin can induce aneuploidy in murine oocytes treated prior to ovulation. Control animals were injected with intraperitoneal (ip) saline and the treatment animals were divided into three different dose schedules for ip cisplatin. After ovulation induction, the oocytes were processed for cytogenetic analysis and the chromosomes were counted. There were significantly less oocytes ovulated in the treatment groups when compared to control animals. There was a significant increase in hypohaploid cells counted in the 7.5 mg/kg treatment group, but the percentages of hyperhaploid and polyploid cells were not increased in the treatment groups. The cytogenetic effects of cisplatin *in vivo* and *in vitro* are reviewed.

INTRODUCTION

cis-diamminedichloroplatinum II (cisplatin) is a commonly used chemotherapeutic agent in the treatment of gynecologic malignancies. Some patients treated with cisplatin may retain their reproductive capabilities and survive their disease. The effects of this cytotoxic agent on the fertility and progeny of the above patients are largely unknown.

Cisplatin is a mutagen and a carcinogen [1]. It produces genetic damage in bacteria [2], *in vitro* cell lines [3], mouse bone marrow cells [4], and human lymphocytes [5]. Cisplatin-induced genetic damage has been shown to result primarily in DNA intrastrand cross-links or adduct formation [6]. This damage occurs in quanine-rich DNA sequences [7], and also inhibits DNA synthesis and replication by inactivating the template. Other observations related to the genetic toxicity associated with cisplatin therapy include damage to microtubule formation [8] and G2 arrest [9].

Studies involving the genotoxic effects of cisplatin on germ cells have largely been done in males. However, male and female germ cells differ in their susceptibility to mutation induction, thus genotoxic data obtained from male germ cells cannot be appropriately extrapolated to female germ cells [10].

Aneuploidy is a distinct indicator of germ cell chromosomal damage. It is the most important cause of spontaneous pregnancy loss and multiple anomalies in the liveborn infant [11]. This study was undertaken to determine if cisplatin can induce aneuploidy in murine oocytes treated prior to ovulation.

MATERIALS AND METHODS

Virgin female mice (Harlan Sprague–Dawley, Inc.) were housed under a 12-hr light/12-hr dark schedule and allowed food and water *ad libitum*. The mice were 12–14 weeks of age and weighed 25–32 g. Follicle maturation was induced by intraperitoneal (ip) injection of 7.5 IU of pregnant mare's serum (PMS, Folligon, Intervet, Ltd., Cambridge). Ovulation was induced 48 hr after PMS injection with ip injection of 5.0 IU human chorionic gonadotropin (HCG, Ayerst, NY).

Cisplatin was dissolved in normal saline and injected ip immediately following HCG injection. Control animals received normal saline ip. The treatment group was divided into three different dosages of cisplatin, 5.0, 7.5, and 10.0 mg/kg, given in a single ip injection immediately after HCG injection.

The doses for the treatment groups were chosen for direct comparison with those doses used in cisplatin experiments involving male germ cells [12]. The LD_{50} of cisplatin in female mice is 13 mg/kg [13], and studies measuring serum levels after ip injection of 7.5 mg/kg of cisplatin in mice [14] are comparable to measured serum levels in humans after intravenous administration of cisplatin at doses of 40–100 mg/M^2 [15].

The mice were sacrificed by CO^2 inhalation 17 hr after

[1] To whom reprint requests should be addressed at Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, LSU School of Medicine, Shreveport, 1501 Kings Highway, P.O. Box 33932, Shreveport, LA 71130-3932.

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TABLE 1
Number of Animals and Oocytes Collected

Cisplatin (mg/kg)	Mice (*N*)	Oocytes collected	Average oocytes per mouse
Control	65	2809	43.2
5.0	105	3636	34.6
7.5	90	2354	26.2
10.0	127	2702	21.3

HCG injection and the cumulus masses were dissected from the oviduct microscopically and placed into Hanks' balanced salt solution (HBSS). The oocytes were then transferred into 150 IU hyaluronidase (Sigma Chemical Co., No. 3506) per ml HBSS for 15 min at room temperature to dislodge the oocytes from the surrounding cumulus. The oocytes were rinsed twice in HBSS and transferred to another well of a spot plate containing 0.3% sodium citrate solution (hypotonic) for 30 min at room temperature. The oocytes were then transferred to a microcentrifuge tube and subjected to a series of gradual fixation steps using 3:1 fixative (methanol:glacial acetic acid). Grease-free microscopic slides were dipped into distilled water and the cellular solution was aspirated into a pulled Pasteur pipette and gently dropped onto the slide. A more detailed description of the above technique was reported by Mailhes and Yuan in 1987 [16]. Chromosomes were then C-banded according to the procedure of Salamanca and Armendares [17].

The numbers of metaphase I and metaphase II oocytes on each slide were recorded. In each oocyte, the number of C-banded chromosomes along with structural aberrations were determined. Cells were characterized as euploid (N = 20), hypohaploid (N = 10–19½), hyperhaploid (N = 20½–29½), and polyploid (N = 30–40). Cells were considered noninterpretable if there was insufficient chromosome banding, overlapped or clumped chromosomes precluding an accurate count, or extensive chromosome scatter.

χ^2 analyses were used for comparing the frequency of hypohaploidy and for the number of oocytes ovulated. However, Fisher's exact test was used for comparing hyperhaploidy because of the low numbers in these groups. P values less than 0.05 were considered statistically significant.

RESULTS

The numbers of animals and oocytes collected in the control and each treatment group are presented in Table 1. There were an average of 43.2 oocytes/animal collected in the control group and an average of 34.6, 26.2, and 21.3 oocytes/animal in the 5.0, the 7.5, and the 10.0 mg/kg treatment groups, respectively. There were significantly less average oocytes collected per mouse in the 7.5 mg/kg and the 10.0 mg/kg groups when compared to the control animals (P = 0.002 and P = 0.000004, respectively).

The number of cells analyzed in each group and the proportion and characterization of all aneuploidy observed is presented in Table 2. The percentage of hypohaploid oocytes was increased in all treatment groups when compared to the control animals, however, this only reached statistical significance in the 7.5 mg/kg group (P = 0.0004). This finding should be taken with caution, however, since hypohaploid cells are not a reliable indicator of aneuploidy because many are derived from technical artifact. The percentages of hyperhaploid and polyploid cells were not significantly increased in the treatment groups over the controls, and structural aberrations were not observed during the course of cytogenetic analysis.

DISCUSSION

The effects of cisplatin on germ cells is of particular interest because of the potential for inducing cytogenetic aberrations. Meistrich *et al.* found that differentiated murine spermatogonia, spermatocytes, and spermatids were all sensitive to cellular death after cisplatin therapy while stem cells were relatively resistant [18]. They also noted an increase in chromosomal aberrations including multiple chromosome breaks and univalents in all male germ cells tested. Others have found that cisplatin produces a clastogenic effect on differentiated mouse spermatogonia

TABLE 2
Observed Aneuploidy in Metaphase II Oocytes

Cisplatinum (mg/kg)	Total cells	Euploid	%	Hypoploid	%	Hyperploid	%	Polyploid	%
Control	240	226	94.2	12	5.0	1	0.4	1	0.4
5.0	496	455	91.7	36	7.3	5	1.0	0	0
7.5	334	277	82.9	48	14.4	7	2.1	2	0.6
10.0	338	304	89.9	31	9.2	1	0.3	2	0.6

with the number of aberrations per cell increasing in a dose-dependent fashion [19]. Studies in primary mouse spermatocytes treated in the zygotene to preleptotene phases of meiosis I were the most sensitive to induction of chromosomal aberrations. However, they found no significant increase in the translocation frequency in stem cell spermatogonia. They concluded that cisplatin induced genetic aberrations during the premeiotic phase of DNA synthesis [12]. Studies involving the mutagenic potential of cisplatin using dominant lethal assays have failed to show an effect on treated male mice [10,20].

Studies in male patients with testicular malignancies have revealed that most patients are rendered azoospermic by cisplatin-based chemotherapy [21–24]. While restoration of spermatogenesis occurs in many patients after cessation of therapy [21,25], not all patients will demonstrate return of fertility [26]. We have found no published reports of increased congenital abnormalities in children fathered by patients who have received cisplatin [21,25,27–29], although analysis of sperm chromosomes has demonstrated an increased incidence of aberrations years after cessation of therapy [30].

Few studies have addressed the genotoxic effect of cisplatin on oocytes. There are several case reports in the literature of human female patients becoming pregnant after cisplatin-based chemotherapy [31–39]. These reports include 13 normal infants and 2 spontaneous abortions. There have been no neonatal abnormalities reported. Several authors have reported the return of normal menses after cisplatin therapy [35,37], while others have presented evidence of permanent gonadal dysfunction in these patients [40].

While the above clinical experience is encouraging, there are not enough data to appropriately counsel patients, and investigations at the cellular level are needed. Katoh *et al.* treated female mice with 2.5–5 mg/kg of cisplatin and found a significant increase in early embryo lethality [10]. Cytogenetic analysis of the first cleavage metaphase zygote showed an increase in structural chromosomal aberrations in the treated animals with preferential damage to the centromeric region noted. The authors proposed that the diffuse state of the oocyte chromatin makes it more susceptible to cisplatin damage than the more condensed male germ cell chromatin, thus explaining the difference in the dominant lethal results between male and female mice.

Our study is of interest because of the lack of aneuploidy observed in oocytes treated during prophase of meiosis I. Although a higher proportion of hypohaploid oocytes was found in the 7.5 mg/kg group, this value probably represents technical artifact secondary to chromosome loss during slide preparation. Some investigators have found that lethal cell damage can occur with the same efficacy in all stages of the cell cycle tested [41], while others have reported that the genetic aberrations observed indicate that the cells have progressed through at least some portion of the S phase during cisplatin exposure and that DNA replication is necessary for the expression of cisplatin-induced damage [5]. The treated cells in our study did not proceed through an S phase before analysis in metaphase II and this could explain the lack of cisplatin-induced effect. Katoh's first cleavage zygote studies above would tend to support this premise [10].

We did not measure the concentration of cisplatin in the follicular fluid in the treated animals, and with the negative findings of this study, the question could be raised concerning the exposure of the treated oocytes to the drug. Studies using ip administration of cisplatin in mice have shown that the α-half-life of cisplatin is 1.5–5 hr, with peak plasma levels measured within 1 hr of ip injection [42]. All animal species studied have demonstrated initial distribution to nearly all organs, and studies involving dogs show that the highest levels are found in the ovary, kidney, liver, uterus, skin, and bone [43]. Katoh *et al.* demonstrated embryonic lethality and chromosomal aberrations when female mice were treated with a single ip injection 12 hr before mating, and these effects persisted when the treated zygotes were transferred to donor females not exposed to cisplatin [10]. Our study showed a decrease in the number of oocytes in the treated mice, and we feel that this effect and the above data indicate that the oocytes in our study were probably exposed to the drug.

The decrease in the number of oocytes ovulated in the treatment groups in our study was an unexpected finding. While it has been demonstrated that cisplatin therapy will decrease the concentration of pituitary hormones [44], these were given exogenously to the animals in our study. Possible explanations would include gonadotropin receptor dysfunction or local effects within the ovary preventing oocyte extrusion, or atresia within the preovulatory follicle. Histologic studies on the cisplatin-treated ovaries would be of interest in future studies.

In conclusion, we found no significant increase in aneuploidy formation in metaphase II oocytes treated during prophase of meiosis I, but there was a significant reduction in the number of oocytes ovulated in the cisplatin-treated animals. First cleavage zygote studies are underway to evaluate whether aneuploidy becomes evident after these cells pass through the S phase of the cell cycle and whether the treated oocytes show impaired fertilization rates and are capable of developing into normal embryos.

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