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IN VIVO TOXICITY OF CISPLATIN AND CARBOPLATIN ON THE LEYDIG CELL FUNCTION AND EFFECT OF THE HUMAN CHORIOGONADOTROPIN

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Abstract—In order to characterize the respective toxicity of cisplatin (CDDP) and carboplatin (CBDCA) on the male reproductive system, we have investigated their *in vivo* effects on the steroidogenesis function of rat leydig cells. Animals were treated at the respective LD_{50} of platin compounds, and we analyzed plasmatic testosterone level, microsomal cytochrome P-450 and platinum concentrations in the testis. CDDP induces a dramatic change in both the testosterone level and the microsomal cytochrome P-450 concentration. In contrast, CBDCA was found to be less toxic than CDDP, probably due to its different accumulation at the testis level. We also investigated the potential action of human chorionic gonadotropin which allows a full restoration of the steroidogenesis function.

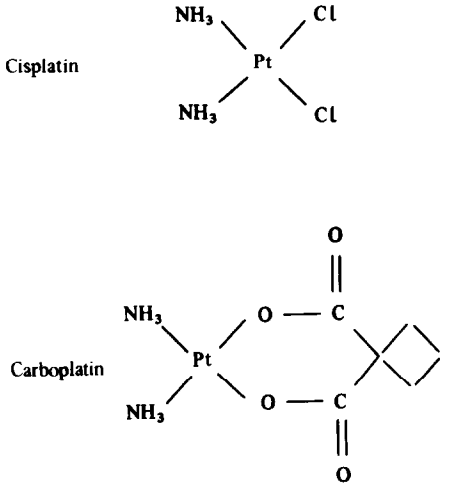

Fig. 1. Structural formulae of cisplatin and derivatives included in this study.

Cisplatin (CDDP, *cis*-diamine dichloroplatinum II)§ is highly efficient against a number of human tumors and has now become a part of the standard regimen for testicular tumors, head and neck carcinoma and ovarian carcinoma [1–3]. However, its full potential is limited by major side effects, including nephrotoxicity, severe nausea and vomiting, ototoxicity, peripheral neuropathy and sterility [2, 4, 5]. Carboplatin (CBDCA, *cis*-diamine-1,1-cyclobutane dicarboxylate platinum II, JM8) is one of the second generation platinum-coordination complexes selected for further clinical studies [4, 6] on the basis of reduced nephrotoxicity in rats and increased antitumor effects in various animal models [7] (Fig. 1).

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§ Abbreviations used: CDDP, cisplatin, *cis*-diamine dichloroplatinum II; CBDCA, carboplatin, *cis*-diamine-1,1-cyclobutane dicarboxylate platinum II, JM8, hCG, human chorionic gonadotropin.

Recent clinical studies have shown that carboplatin is a very active agent in the treatment of small cell lung cancer [8], head and neck carcinoma [4], ovarian carcinoma [6], breast carcinoma [6], testicular teratoma and testicular seminoma [9].

Cisplatin is currently used in the treatment of testicular cancer which has been proved to be one of the better targets of the drug. As a consequence of its site of action, cisplatin exerts damages on the normal functions of the testis. Rubery [5] reported transient sterility in man after treatment with cisplatin. Vawda *et al.* [10] have shown that cisplatin treatment reduces the number of primary spermatocytes in the mouse. Maines *et al.* [11] proved that cisplatin treatment decreases the plasma testosterone concentration and the microsomal cytochrome P-450 levels in the rat testis. These results might explain the sterility observed during the treatment with cisplatin. Further, these authors [11] demonstrated that human chorionic gonadotropin (hCG) partially restores the cytochrome P-450 concentration and allows the plasma testosterone level to approach control values after treatment with cisplatin.

In order to characterize further the respective toxicity of cisplatin and carboplatin on the male reproductive system, we have investigated their *in vivo* effects on the testis functions and particularly on the steroidogenesis function of the leydig cells. In the present report, we analysed both the plasma testosterone concentration, the cytochrome P-450 level and the platinum distribution in rat testis during the course of the treatment with cisplatin and carboplatin. Protective effect of hCG was also investigated.

MATERIALS AND METHODS

Cisplatin was obtained from Lilly Laboratories (Saint-Cloud, France); carboplatin was a gift from Bristol Laboratories (Paris, France); human chori-

Table 1. Treatment schedules and platinum concentration in the testis and in the serum*

Group	Product	Dose	Route	Schedule	Platinum Testis†	Platinum Serum‡
I	Mannitol	100 mg/kg	i.v.	day 1	<3	<3
II	Cisplatin	9 mg/kg	i.v.	day 1	83 ± 12	26 ± 6
III	Cisplatin	9 mg/kg	i.v.	day 1		
	hCG	500 IU/kg	s.c.	days 1 to 6	86 ± 15	33 ± 6
IV	Carboplatin	60 mg/kg	i.v.	day 1	52 ± 6	27 ± 4
V	Carboplatin	60 mg/kg	i.v.	day 1		
	hCG	500 IU/kg	s.c.	days 1 to 6	63 ± 10	23 ± 3
VI	hCG	500 IU/kg	s.c.	days 1 to 6	<3	<3

* Platinum determination in the serum samples and testis homogenates was performed as described under Materials and Methods. Each value is the mean ± SEM of results obtained from four animals and each measurement was performed in triplicate.

† Results expressed in ng/g of organ weight.

‡ Results expressed in ng/ml of serum.

onic gonadotropin (hCG)-Pregnyl was obtained from Organon Laboratories (Serifontaine, France); Bio-Rad protein assay kits were obtained from Bio-Rad Laboratories (München, F.R.G.). All the other compounds were of the best grade available and were obtained from commercial sources.

Animals and treatments. Twenty-four male Wistar rats weighing 180–200 g were conditioned for five days prior to their use in the study. Food and water were given *ad libitum*. They were divided into six groups of four rats each. Blood samples were collected for basal testosterone measurement one day before the treatment. Then, the different groups were treated as reported in Table 1. Rats were treated with cisplatin and carboplatin at the LD_{50} (respectively 9 mg/kg and 60 mg/kg) which induces a 50% lethality between days 10 and 14 after treatment. No deaths were observed at day 7. On days 3 and 7, blood samples were collected for the testosterone determination. On day 7, the rats were withdrawn for both testosterone and platinum determination and killed. The testis were removed immediately, washed in ice-cold NaCl (0.9%), weighed and frozen in liquid nitrogen. At the same time, all blood samples were centrifuged, blood serum and plasma were frozen at −20° before use.

Steroid analysis. The plasma testosterone level was determined by a radioimmunoassay (Bio-Mérieux, Marcy-L'Etoile, France). The cross-reactivity of the anti-testosterone serum is about 48% for 5α-dihydrotestosterone and less than 0.1% for the other steroid hormones.

Cytochrome P-450 determination. The testis were homogenized at 4° in a Teflon–glass homogenizer in 5 vol. of a buffer containing Tris 1 mM, sucrose 0.25 M and EDTA 1 mM, pH 7.5 [12–15]. One millilitre of each homogenate was collected for platinum assay. The homogenates were then centrifuged (800 *g*, 10 min, 4°). The supernatants were collected and centrifuged (9000 *g*, 30 min, 4°) in a Beckman L8-70 Ultracentrifuge. The resulting supernatants were subsequently centrifuged (105,000 *g*, 1 hr, 4°). The pelleted microsomes were washed once with a 100 mM sodium pyrophosphate buffer, pH 7.4, and resuspended in 0.5 ml of 100 mM sodium phosphate buffer, pH 7.5, containing 20% glycerol (Buffer A). The protein content was determined by the Bio-Rad method using bovin serum albumin as the standard.

The cytochrome P-450 determination was performed by analysis of the carbon monoxide (CO) absorption spectrum using a method adapted from Nozu *et al.* [14] and Omura *et al.* [16]. The reduced-CO difference spectrum of the suspension was obtained using sodium dithionite as the reducing agent. The microsomal preparation was suspended in the Buffer A and sodium dithionite was added to the suspension which was equally divided between two cuvettes with an optical path of 1 cm. One cuvette was slowly bubbled with CO for 20–30 sec, and the CO-difference spectrum was recorded in a Kontron-Uvikon 860 spectrophotometer. The concentration of cytochrome P-450 was calculated using an extinction coefficient of 91 $mM^{-1} \cdot cm^{-1}$ for the absorption difference between 450 and 490 nm.

Statistical analysis of the results of steroid and cytochrome P-450 determinations was carried out by analysis of variance and Student's *t*-test.

Platinum measurement. Platinum assay in blood serum and tissue samples [17–21] was performed using a Perkin-Elmer 560 atomic absorption spectrophotometer (AS-1 injector and HGA 500 graphite furnace equiped with pyrocoated tubes), according to the technique described by Le Roy *et al.* [20]. Samples of blood serum (1 ml) and testis homogenate (1 ml) were digested in 3 ml of concentrated (14 N) nitric acid at 50° for 1 hr. When the dissolution was achieved, the nitric acid was evaporated to dryness and the residue was then solubilized in 0.1 N hydrochloric acid (0.5 ml). Platinum concentrations were calculated from a standard curve using a six stages temperature program as follows: 80 sec at 100° to dry the sample, 20 sec at 140° to char the sample, 20 sec at 400° to precipitate the proteins, 30 sec at 1400° to have metal platinum (Pt_0), and two final stages of 4 and 2 sec respectively at 2700° to get the atomization of the platinum. Absorption was measured at 265.9 nm.

RESULTS

Effect of cisplatin and carboplatin on plasma testosterone level

Using cisplatin and carboplatin at the LD_{50} [22],

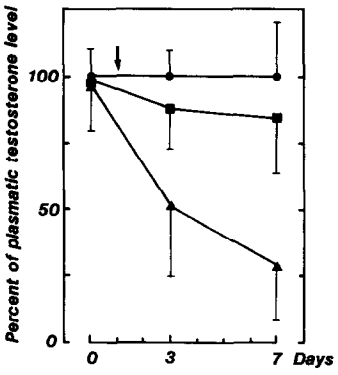

Fig. 2. Effect of cisplatin and carboplatin treatments on the plasmatic testosterone level: male Wistar rats (180–200 g) were treated intravenously either with mannitol (100 mg/kg) control group (●—●), or cisplatin (9 mg/kg) (▲—▲) or carboplatin (60 mg/kg) (■—■). Plasma samples were collected at day 0, day 3 and day 7 (after injection); the testosterone concentration was determined as described under Materials and Methods. Results are expressed as the mean ± SEM of four separate values, and are calculated in comparison to the control group (percentage of treated versus control animals). Control values of plasmatic testosterone level were 3.93 ± 0.20 ng/ml of plasma.

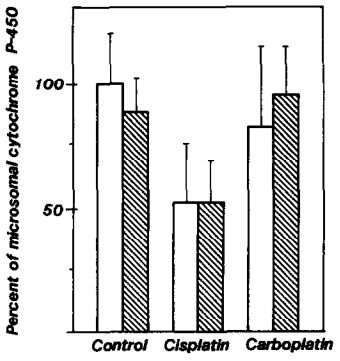

Fig. 3. Effect of cisplatin, carboplatin and hCG treatments on the testicular cytochrome P-450 concentration. Three groups of Wistar rats (180–200 g) were treated once intravenously either with mannitol (100 mg/kg) for the control group or cisplatin (9 mg/kg) or carboplatin (60 mg/kg: open bars □. Three other groups of Wistar rats (180–200 g) were given hCG, in addition to the precedent treatments, once a day subcutaneously (500 IU/kg for 7 days: hatched bars (▨). The animals were killed 7 days after the first injection. The microsomal fractions were prepared and used for determination of microsomal cytochrome P-450 in the testis. The methods used for the preparation of tissue fractions and assay procedures are provided in detail under Materials and Methods. The data shown are the per cent change compared to controls of cytochrome P-450 concentration in the testis. The results are expressed as the mean ± SEM of four determinations. The control values for testicular cytochrome P-450 concentration were 34 ± 7 pmoles of cytochrome P-450/mg of proteins.

we observed that cisplatin treatment induced a significant change in the plasma testosterone level (Fig. 2). A 50% decrease on day 3 ($P < 0.001$) and 70% decrease on day 7 ($P < 0.001$) were observed in comparison to the control group that received a single injection of mannitol. Treatment of rats with carboplatin decreased the plasma testosterone level to 12% and 15% of the control value on days 3 and 7 respectively, but this change was not significant as evaluated by the Student's *t*-test.

Effect of cisplatin and carboplatin on the microsomal cytochromes P-450 concentration in the rat testis

Microsomal cytochrome P-450 contents were determined in rat testis after treatment with both cisplatin compounds. The results, shown in Fig. 3, are expressed in per cent of the control values. It appears that cisplatin induces a significant decrease (50%) in the microsomal cytochrome P-450 concentration ($P < 0.02$). In contrast, no significant decrease (20%) was observed with the carboplatin regimen.

Similar results on the biochemical parameters were observed in two sets of experiments: data statistically evaluated by analysis of variance did not demonstrate any significant differences between the two sets.

Effect of hCG on both biochemical parameters

A large restoration of the plasmatic testosterone level was observed when hCG was infused to animals treated with either cisplatin or carboplatin (Fig. 4). It is striking to notice that the concentration of plasma testosterone in rat treated with cisplatin was largely affected by the administration of hCG as its level increased from 1.20 ng/ml to 19.70 ng/ml on day 7. Such change was also observed in rat treated with carboplatin; testosterone level was increased

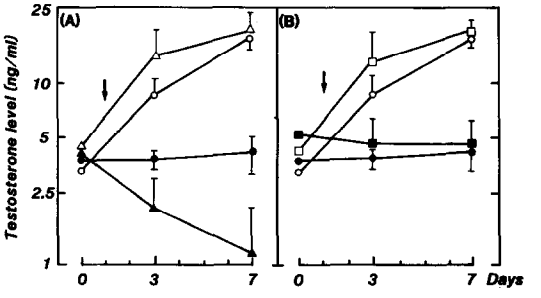

Fig. 4. (A) Effect of hCG on the plasma testosterone level in rats treated with cisplatin. Four groups of Wistar rats (180–200 g) were treated intravenously either with mannitol (100 mg/kg) for the control group (●—●), with cisplatin (9 mg/kg (▲—▲), with cisplatin (9 mg/kg) plus hCG (500 IU/kg of weight, subcutaneously for 7 days (△—△), or hCG alone (500 IU/kg of weight, subcutaneously for 7 days (○—○). Plasmatic testosterone level was determined at days 0, 3 and 7 after the first injection, according to the method described under Materials and Methods. Results are expressed as the mean ± SEM of four determinations in a single experiment. (B) Effect of hCG on the plasma testosterone level in rats treated with carboplatin. Four groups of Wistar rats (180–200 g) were treated intravenously either with mannitol (100 mg/kg) for the control group (●—●), with carboplatin (60 mg/kg (■—■), with carboplatin (60 mg/kg) plus hCG (500 IU/kg of weight, subcutaneously for 7 days (□—□) or hCG alone (500 IU/kg of weight, subcutaneously for 7 days) (○—○). Plasmatic testosterone level was determined at days 0, 3 and 7 after the first injection, according to the method described under Materials and Methods. Results are expressed as the mean ± SEM of four determinations.

from 4.44 ng/ml to 18.96 ng/ml on day 7. In both cases, the plasmatic testosterone level was similar to that observed when rats were treated with hCG alone. Further, we have not detected any significant change in the microsomal cytochrome P-450 concentration after hCG administration.

Distribution of cisplatin and carboplatin in rat testis and serum

Table 1 depicts the platinum distribution in the serum and testis of rats treated with cisplatin and carboplatin. After 7 days of treatment, the serum of the rats presented an accumulation of platinum which was nearly the same for both cisplatin and carboplatin treatments (26 ± 6 ng/ml and 27 ± 4 ng/ml respectively). At the testicular level, platinum concentration observed after cisplatin treatment was higher than that reported after carboplatin treatment. Concentrations were about 80 ng of platinum per g of tissue for cisplatin and about 50 ng of platinum per g of tissue for carboplatin. Similar concentrations were observed when hCG was added to the treatment regimen.

DISCUSSION

Cisplatin is an effective chemotherapeutic agent against a broad spectrum of human malignancies including testicular cancer. Male sterility is one of the side effects of the treatment by cisplatin as it has been reported in several clinical observations [5]. Carboplatin, a new platinum complexes coordination, shows a comparable efficacy on the testicular cancer [9]. Thus, our study was undertaken to explore the *in vivo* toxicity of cisplatin and carboplatin on the Leydig cell function. Plasmatic testosterone level, microsomal cytochrome P-450 concentration and platinum distribution in the testis were analysed. To this end, a single injection of each platinum compounds at the LD_{50} was administered to rats and toxicities were evaluated at days 3 and 7. The protective effect of hCG on both drugs treatment was also investigated. In the present report, we demonstrated that carboplatin induces a weak toxicity on the Leydig cell function in contrast to cisplatin which produces a depletion of 70% of the plasma testosterone level and of 50% on the microsomal cytochrome P-450 concentration in the rat testis. Carboplatin treatment induced a decrease of about 15% in the plasma testosterone level and of 20% on the microsomal cytochrome P-450 concentration: such a decrease was found to be not significant.

Both CDDP and CBDCA are converted to the same diaquated species intracellularly which alkylates DNA; therefore, the mechanism of cytotoxicity of these drugs is the same. Thus, differences in the toxicity of carboplatin versus cisplatin may be due to a different pattern of distribution and affinity to the considered organ. We analyse the distribution of platinum after a single injection of carboplatin; the testis platinum concentration represents only the two-thirds of that obtained after cisplatin injection. It is noteworthy that the observed plasma concentration of platinum is nearly similar for the two drugs, although the quantities of drugs administered to the animals are quite different. These results are explained by the differences in the pharmacokinetic parameters of both compounds as reported previously [23, 24]. In fact, as a consequence of their respective chemical structures and stabilities [25, 26], the two drugs undergo different features in their elimination phase, more rapid for carboplatin than for cisplatin, their plasmatic protein binding [27], their tissue distribution [28] and their renal excretion [26, 29]. Our data show that carboplatin accumulates in less extent than cisplatin in the testis as measured seven days after a single injection. It is likely that the weak accumulation of carboplatin explains the small changes induced by the drug on the biochemical parameters analysed in our study.

hCG binds to high affinity receptors located on the plasma membrane of testicular leydig cells and stimulates steroidogenesis [14]. Several steps in the biosynthesis of steroids are catalyzed by cytochrome P-450 which have been reported as the target of several drugs [11]. Thus, we investigated the effect of hCG on cisplatin and carboplatin toxicities on the leydig cell steroidogenesis. Our data suggest that hCG could protect the steroidogenesis function of the leydig cells as we found a large restoration of the plasmatic testosterone level after both treatment with cisplatin or carboplatin. In contrast to the results observed by Maines *et al.* [11], we did not denote a significant restoration of the microsomal cytochrome P-450 concentration in the rat testis. We relate this observation to the dose of hCG used in our experiment. In fact, earlier studies [30] suggest that, using high doses of hCG, the cytochrome P-450 level declines by 2 days after treatment, returns to normal values by 4 days and does not show significant augmentation before 14 days. So, as we sacrificed the animals on day 7, complete restoration and increase in the cytochrome P-450 level might not be detected. Furthermore the high level of plasmatic testosterone might induce a negative regulation on the cytochrome P-450 concentration as it has been described elsewhere [31].

Taken together, our results demonstrate that carboplatin is less toxic than cisplatin on the steroidogenesis function of the leydig cells. hCG appears to be an effective protector of the testosterone biosynthesis after drugs treatment, while its mechanism of action remains unclear.

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